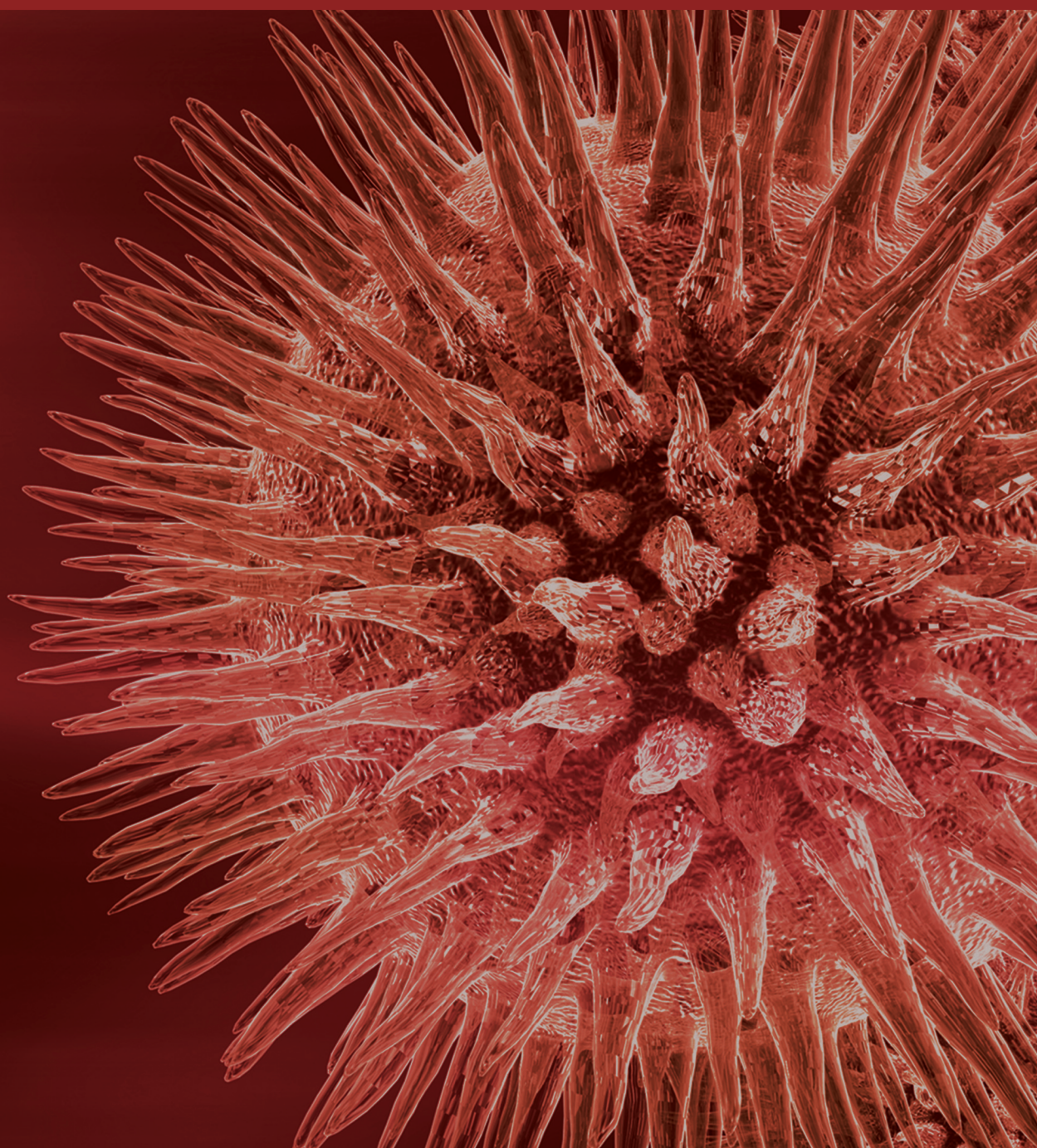


New Advances in Reproductive Biomedicine

Guest Editors: Irma Virant-Klun, Jeroen Krijgsveld, John Huntriss,
and Raymond J. Rodgers





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BioMed Research International

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Editorial

New Advances in Reproductive Biomedicine

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Since birth of the first “test tube” baby—Louise Brown—in 1978, pioneered by physiologist Robert G. Edwards and surgeon Patrick C. Steptoe, there was a great advance in reproductive biomedicine made by amazing people, who insisted on this path in spite of pressure from society. In vitro fertilization procedure indeed became an established way to treat couples with severe infertility problems, and until today more than four million babies have been born worldwide in this way. The field of assisted conception is a fast-developing domain at both the basic and clinical level. This is reflected in the 31 papers in this special issue, spanning a wide range of topics including female and male infertility, gametes, embryos, pregnancy, and babies born after assisted conception.

There are some new aspects to study and think about female (in)fertility. The methodology of proteomics was exposed to be important in potential linking oxidative stress and female infertility (S. Gupta et al.). One of the main tasks in the in vitro fertilization program remains to better elucidate oocyte quality. This can be facilitated by novel proteomic and secretome tools as have been used in animal models, although some obstacles still need to be resolved before these can be applied to human cells (I. Virant-Klun and J. Krijgsveld). The quality of oocytes also depends on mutual interactions with surrounding granulosa cells; therefore, global profiling of gene expression of follicular cumulus cells using microarrays and next generation sequencing (NGS)

may lead to establishment of new reliable biomarkers to select oocytes with the highest developmental potential (E. Chronowska). Oocyte maturity is a prerequisite for successful fertilization and pregnancy. Time-lapse dynamics of oocyte chromatin organisation during meiotic resumption was observed in a mouse model, providing a pathway of transition from the GV to the MII stage oocyte (M. Belli et al.). In vitro maturation (IVM) of human oocytes is still a suboptimal procedure resulting in impaired oocyte developmental potential. However, results on bovine oocytes showed that addition of hyaluronan (HA) to the IVM medium improved the quality of in vitro derived embryos-blastocysts, which expressed less DNA fragmentation (J. Opiela et al.). Oocyte quality can be influenced by female infertility factors. In nonoverweight women with polycystic ovary syndrome (PCOS), the association between follicular fluid leptin and serum insulin was confirmed (G. Garruti et al.). It was proposed that lower levels of leptin in follicular fluid may explain the lower oocyte quality and fertilization in these women and this may be related to the level of insulin and/or insulin resistance.

Most instances of severe infertility in men cannot be explained. A special concern is represented by male and couple fertility impairment due to human papilloma virus (HPV) DNA sperm infection (S. Gizzo et al.). The question of potential negative impact of HPV infection on semen quality is still open, despite high prevalence of infection (37.1%) in

men from infertile couples (B. Golob et al.). In nonobstructive azoospermia, the increase in volume density of both the CD 68-positive macrophages and vacuolated Leydig cells and a positive correlation between the volume densities of these cell types were observed; it was proposed that overstimulation of Leydig cells, together with a negative paracrine action of macrophages, could result in the damage of steroidogenesis and lower levels of testosterone in situ (T. Goluza et al.). The fertilization potential of spermatozoa depends on appropriate and time-dependent process of hyperactivation, chemotaxis, capacitation, and the acrosome reaction, where calcium (Ca^{2+}) is extensively involved in almost every step. Modern proteomic analyses have identified several sperm proteins, and therefore these findings might provide further insight into understanding the Ca^{2+} influx, protein functions, and regulation of fertility (M. S. Rahman et al.).

At present there is still no generally accepted clinical treatment to improve the semen quality in infertile men, although some clinical trials showed that this may be improved by oral administration of antioxidants (S. Imamovic Kumalic and B. Pinter). Furthermore, a special concern are also the paternal, sperm-borne mitochondria (mt) that are selectively degraded inside the fertilized oocytes. Assisted reproductive therapies (ART) such as intracytoplasmic sperm injection (ICSI) for male infertility treatment include the injection of the entire sperm into oocytes including mitochondria and mtDNA; therefore, sperm mitochondrial degradation mechanisms may play a crucial role with implications to health, fitness and fertility, and development and health of ART babies (W.-H. Song et al.).

Array comparative genomic hybridization (aCGH) for comprehensive screening of all chromosomes is becoming a new tool for selection of euploid embryos to be transferred in the in vitro fertilization program (L. Rodrigo et al.) and can improve the clinical outcome—pregnancy rate—in women with repeated implantation failure (E. Greco et al.). Yet, some caution is needed, because embryo biopsy is an invasive procedure, and less invasive procedures to distinct normal and abnormal embryos need to be developed (T. Vladimirova Milachich). Not only embryo but also the endometrium plays an important role in successful implantation. One of the first studies confirmed that the infusion of granulocyte colony-stimulating factor (G-CSF) leads to the improvement in endometrium thickness and the chance to achieve pregnancy in women with treatment-resistant thin endometrium (M. Kunicki et al.). In a clinical study, tissue Doppler imaging (TDI) based elastography was used for assessment of cervical stiffness during all three trimesters of pregnancy and a correlation with gestational age, cervical length, and parity was found (A. Fruscalzo et al.). Fetal middle cerebral artery (MCA) circulation was followed after defined prenatal acoustical stimulation (PAS), correlating the role of cilia in hearing and memory in fetus to signal transduction (i.e., optical-acoustical properties). The authors suggest that fetuses are getting used to sound, developing a kind of memory patterns, considering acoustical and electromagnetic waves (S. Jankovic-Raznatovic et al.). Further, BACs-on-beads technology represents a new and

reliable test for rapid detection of fetal aneuploidies and microdeletions in prenatal diagnostics (S. García-Herrero et al.). Quantitative proteomics analysis revealed the altered protein expression in the placental villous tissue of early pregnancy loss using isobaric tandem mass tags and may represent a new diagnostic tool in the future (X. Ni et al.).

Besides in vitro fertilization itself, cryopreservation of embryos, gametes, and reproductive tissues represents an extremely important part of assisted conception to preserve the potential of life and fertility, as reviewed by J. Konc et al. Advanced experimental data were provided on new approaches to human ovarian tissue cryopreservation in young cancer patients, showing vitrification with good preservation of stromal cells without apoptosis in human ovarian tissue (R. Fabbri et al.) and an effective in vitro perfusion of ovine intact ovaries with vascular pedicle by freezing medium, important for development of whole ovary cryopreservation in humans (V. Isachenko et al.). An option in cancer patients is also to cryopreserve the oocytes, if ovarian stimulation is safe and possible, but this is not an easy task and was thoroughly reviewed in bovine oocytes (I.-S. Hwang and S. Hochi). The data also show that superoxide dismutase (SOD) represents a good predicting factor for boar semen characteristics after short-term cryostorage (M. Zakosek Pipan et al.) and may be applied in human medicine.

Each birth of a baby after in vitro fertilization still represents a great effort and a “miracle.” During the last years, several studies have shown that babies after assisted conception are healthy and do not differ from other babies. Nevertheless, permanent concern is needed for both mother and baby to be safe and healthy. In a clinical study, singleton pregnancy and neonate outcomes after in vitro fertilization with fresh or frozen-thawed embryo transfer were followed in a large group of women (S. Korosec et al.).

The perspectives and challenges of pluripotent and induced pluripotent stem cells as new agents for treatment of infertility are discussed by V. Volarevic et al. Moreover, stem cells from follicular aspirates in infertile women were successfully cultured in vitro. They expressed several genes related to mesenchymal stem cells and were successfully differentiated into cells of all three germ layers (E. Dzafic et al.). These cells may be interesting for both the reproductive (e.g., regeneration of ovaries) and regenerative medicine. Also spermatogonial stem cells were further researched by generating gene expression profiles for human spermatogonia both after short- and long-term culture (S. Conrad et al.).

In this special issue, it is also reported that full reimbursement of in vitro fertilization with repeated attempts can provide a notable success considering cumulative delivery rate and should thus be proposed to public health systems (U. Vrtacnik et al.). All great efforts summarized in this special issue not only bring several scientific and clinical answers but also open some new questions, which are an important task and an exciting challenge for the future.

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Review Article

Regulation of Mitochondrial Genome Inheritance by Autophagy and Ubiquitin-Proteasome System: Implications for Health, Fitness, and Fertility

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Mitochondria, the energy-generating organelles, play a role in numerous cellular functions including adenosine triphosphate (ATP) production, cellular homeostasis, and apoptosis. Maternal inheritance of mitochondria and mitochondrial DNA (mtDNA) is universally observed in humans and most animals. In general, high levels of mitochondrial heteroplasmy might contribute to a detrimental effect on fitness and disease resistance. Therefore, a disposal of the sperm-derived mitochondria inside fertilized oocytes assures normal preimplantation embryo development. Here we summarize the current research and knowledge concerning the role of autophagic pathway and ubiquitin-proteasome-dependent proteolysis in sperm mitophagy in mammals, including humans. Current data indicate that sperm mitophagy inside the fertilized oocyte could occur along multiple degradation routes converging on autophagic clearance of paternal mitochondria. The influence of assisted reproductive therapies (ART) such as intracytoplasmic sperm injection (ICSI), mitochondrial replacement (MR), and assisted fertilization of oocytes from patients of advanced reproductive age on mitochondrial function, inheritance, and fitness and for the development and health of ART babies will be of particular interest to clinical audiences. Altogether, the study of sperm mitophagy after fertilization has implications in the timing of evolution and developmental and reproductive biology and in human health, fitness, and management of mitochondrial disease.

1. General Introduction to the Origin of Mitochondria and Unique Features of the Mitochondrial Genome

1.1. Mitochondria and Their Origin. Mitochondria exist in almost all eukaryotic cells. They are semiautonomous, having their own genome and their transcriptional and protein synthesizing machinery [1]. Mitochondria play an important role in numerous cellular functions including calcium signaling, programmed cell death (apoptosis), cellular aging, and energy generation. They generate cellular adenosine triphosphate (ATP) and control the machinery for cellular differentiation, cell death, and cell cycle [2].

The origin of mitochondria from a bacterial symbiont has been widely accepted. The most frequently cited hypothesis to explain the origin of mitochondria is the endosymbiosis theory proposed by Margulis [3]; it states that mitochondria descended from free-living eubacteria, which we know now, have their own DNA and functioning protein synthesis system. The Margulis theory, harshly criticized at the time, postulated that the nucleus came from an archaeobacterium and the symbiotic relationship began with an eubacterial progenitor of the modern mitochondria [4].

The origin of the mitochondrial lineages is associated with increasing oxygen levels in the atmosphere. The consumption of oxygen by metabolism produces energy in the

form of ATP. It was previously believed that the symbiosis between the host and the endosymbiont was based on the endosymbiont producing ATP and the host cell was using carbohydrates exchanged by ATP. However, anaerobic ATP-producing mitochondria are present in unicellular protists and nematodes. They depend on NO_2^- and NO_3^- rather than O_2 . Hydrogenosomes, which are another type of anaerobic ATP-producing organelle, are linked to H_2 for ATP production, harboring O_2 -sensitive enzymes including pyruvate:ferredoxin oxidoreductase and hydrogenase [5, 6].

1.2. Genes Encoded by mtDNA and Their Function. In most eukaryotic cells, mitochondrial DNA (mtDNA) is composed of a circular, double-stranded DNA, where the inner circle represents the cytosine-rich light strand (L-strand) and the outer circle represents the guanine-rich heavy strand (H-strand). The mitochondrial genome in mammals encodes 37 genes: 13 protein coding, 22 mitochondrial tRNA coding, and two genes coding 12S and 16S rRNAs. The mtDNA contains a non-coding region, referred to as the D-loop in mammals or the A + T rich region in other organisms such as insects, in which the mitochondrial transcription promoter is situated. In addition, mtDNA replication also starts in the D-loop. The mitochondrial genetic code is different from nuclear DNA; AGA and AGG are read as stop codons; AUA and AUU are start codons in mitochondrial genes [7]. The major function of genes encoded by mtDNA is the production of core proteins essential for oxidative phosphorylation.

Oxidative phosphorylation in mitochondria is the metabolic pathway in which ATP is generated from electron transport chain located in the mitochondrial inner membrane space [8]. There are five complexes of the electron transport chain: respiratory complex I (NADH: ubiquinone oxidoreductase or NADH dehydrogenase, encoded by *Nad* genes), complex II (succinate: ubiquinone oxidoreductase), complex III (ubiquinol: cytochrome c oxidoreductase), complex IV (cytochrome c oxidase), and complex V (ATP synthase) [9]. Complex I consists of 45 subunits including 14 core subunits and 31 accessory subunits; 7 core subunits are encoded by mtDNA and 7 core subunits encoded by nuclear DNA in bovine [10]. Complex II is composed entirely of nDNA-encoded subunits. Complex III consists of one mtDNA-encoded subunit and 11 nDNA-encoded subunits [7]. Complex IV contains three mtDNA-encoded respiratory chain subunits while the remaining 11 are nuclear DNA [11]. In complex V, only 2 out of 19 subunits are coded by mtDNA [12]. In human mtDNA, complex I discards electrons by NADH, whereas complex II gathers electrons. The electrons are moved to coenzyme Q by both complexes and flow through complex III, then to complex IV to produce water. The electron chemical ingredient that is generated by electron transport chain is utilized for complex V to produce the energy source, ATP [13].

1.3. mtDNA Differs from Nuclear DNA. Nuclear DNA is organized into bead-like structure, the nucleosomes that contain DNA wrapped around core histones and are cross-linked with linker histone H1. The nucleosome consists of two sets of four different histone proteins including H2A, H2B, H3, and H4,

and 170 base pairs of DNA, forming a coiled structure. Histones have the basic DNA-binding proteins that control multiple aspects of DNA function [14]. In contrast to nuclear DNA, mtDNA is not afforded protection and structural organization conveyed by histones. The rRNA and tRNA coding portion of mtDNA chain is packaged into histone-free nucleoids [15] by proteins, most abundantly the mitochondrial transcription factor A (TFAM). The TFAM acts as a protective and regulatory packaging of mtDNA, protecting it from oxidative damage and promoting transcriptional initiation [16]. Interestingly, our previous study on porcine spermiogenesis has provided evidence that sperm TFAM protein is ubiquitinated and segregated from the mitochondria of elongated spermatid to nonmitochondrial region, the principal tail piece of fully differentiated boar spermatozoa. The ubiquitination of TFAM protein in spermatozoa could mediate the recognition of paternal mitochondria by ooplasmic protein/organelle degradation machinery and preclude the transcription of paternal mitochondrial genes in the fertilized oocyte [17].

Nuclear DNA also differs from mtDNA in its structure. Cytological examination reveals that the chromosomal DNA found in the nucleus of most eukaryotes is linear. Consequently, the size and form of chromosomal DNA are considered invariant across generations and tissues [18]. In contrast, mtDNA is circular in most eukaryotes. An exception to this rule is *Schizosaccharomyces pombe*, in which 1% or less of mtDNA is circular and the majority of mtDNA molecules exist in linear and branched forms [19].

Another characteristic differentiating mtDNA from chromosomal DNA is its stability. Nuclear DNA is stable, whereas mtDNA displays significant instability. Induced DNA damage can negatively affect transcriptional regulation, as well as causing mutations due to the change of hydrogen bonding site, such as thymine glycol and 2-hydroxyadenine. Oxidative damage to DNA is the major source of mutation in eukaryotes. The oxidative DNA damage is higher in mtDNA than in nuclear DNA. Mitochondrial DNA is relatively more vulnerable to damage and displays mutations at a 10- to 50-fold higher rate compared to the nuclear genome [20, 21]. In *Drosophila*, the overall mtDNA mutation rate is 10 times higher than that of nuclear DNA [22]. The high rate of mtDNA mutation is posited to be due to its proximity to sites of reactive oxygen species (ROS) production, the lack of protective histones, and limited DNA repair ability; there are, however, two recognized mtDNA repair systems in mammalian cells, the nucleotide excision repair pathway and the base excision repair pathway [23]. This high rate of mutation results in the somatic accumulation of mutations as an organism ages [24].

Mitochondria are now known to be far more dynamic than was thought few years ago, both in their number and localization within a cell, and with regard to copy number. Typically there is a single copy of each nuclear chromosome in each cell. In contrast, there is substantial variability in cellular mtDNA copy number between different organisms and cell types, typically ranging from less than one hundred to several thousands of mtDNA copies per cell [7]. The copy number of mtDNA also changes with exercise [25]. Furthermore, mitochondrial function is linked with the expression of

genes encoded by nuclear DNA; the nuclear genome responds to mitochondrial dysfunction by changes in nuclear gene expression, including but not limited to genes in ubiquitin-proteasome system [26] which, as will be discussed later, contribute to the regulation of mitochondrial inheritance. At the subcellular/organelle level, mitochondria respond to physiological and pathological stimuli by changes in the rate of fusion/fission, shape, and subcellular localization and, when damaged or outlived, are subject to selective removal [27].

Other unique features of mtDNA include the lack or rare occurrence of recombination [28, 29] and the predominantly maternal inheritance in most animal taxa [15]. This uniparental pattern of mtDNA inheritance is called clonal or maternal inheritance. Although maternal inheritance of mtDNA is predominant in eukaryotes, there are different variations of it and we discuss these in the next section.

2. Mechanism of mtDNA Inheritance in Animal Sexual Reproduction

2.1. Parental Modes of mtDNA Inheritance. The inheritance of mitochondrial genome differs from nuclear genomes with biparental inheritance, as the inheritance of mtDNA does not follow a Mendelian pattern. Consequently, mtDNA is strictly inherited from mitochondria of the mother's oocyte in most animals. Some organisms inherit only maternal or paternal mitochondrial genes while others get them from both parents. For example, MtDNA is biparentally inherited in the yeasts *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces* [30].

Over the years, scientists have studied mtDNA inheritance patterns in a variety of organisms. Paternal inheritance of mtDNA occurs in mussels [31], including the families Mytilidae (sea mussels) and Unionidae (fresh water mussels), which have two different types of mtDNA, the F type and the M type. The F type mtDNA is transmitted to the maternal lineage and the M type mtDNA through paternal lineage. This inheritance mechanism has been called doubly uniparental inheritance (DUI). The inheritance pattern of mitochondrial genome is more variable in the interspecific crosses between two species of mouse, *Mus musculus* and *M. spretus*, used to study the mechanism of mitochondrial inheritance in mammals [32]. Due to the failure of sperm mitophagy after fertilization, molecules of paternal mtDNA were detected in hybrid *M. musculus* × *M. spretus* embryos by nested PCR method. Paternal, sperm-derived contingent of mtDNA was estimated at 1–4 molecules per 100,000 maternal, oocyte derived mtDNA molecules. Paternal lineage of mtDNA was also found in an interspecific cross between *Drosophila simulans* and *Drosophila mauritiana* [33], and in the heteroplasmic *D. simulans* lines [34]. It appears that sperm mitochondria are not recognizable to ooplasmic mitophagy machinery in the interspecific crosses, and occasionally, or in some species and lineages regularly, a small amount of paternal mtDNA escapes degradation in the oocyte cytoplasm of interspecific crosses [35].

Although paternal inheritance occurs in mussels and interspecific crosses of *Drosophila* and mouse, paternal mitochondria and their mtDNA cargo is selectively eliminated after fertilization in most animals. Therefore, maternal inheritance of mtDNA is regarded as the major rule of mtDNA transmission in animals and humans.

2.2. Genetic Bottleneck during Gametogenesis. With the exception of interspecific crosses in animals and rare leakage of paternal mtDNA reported in humans [36], the mammalian mitochondrial genome is maternally inherited. However, due to the mutation-prone nature of mtDNA, a mixture of wild-type and mutated mtDNA can be transmitted from mother to progeny, resulting in a condition of two mitochondrial genomes coexisting in the same individual, termed heteroplasmy. High levels of heteroplasmy from maternally transmitted mtDNA mutations are thought to cause mitochondrial disease and have abnormal phenotypes [37, 38]. The mitochondrial bottleneck theory explains the change in mtDNA mutant levels, which indicates a dramatic reduction in mtDNA during embryonic development. The bottleneck is present between the development of primordial germ cell and primary oocyte. The number of replicating mtDNA is increased in the primordial oocyte. Early on, the mitochondrial bottleneck was considered to have a negative effect, causing mitochondrial disorders in progeny. However, it now appears that the mtDNA bottleneck prevents accumulation of adverse mtDNA mutation in the maternal germ line, thus protecting the species by reducing the transmission of mutated mitochondrial genes [39, 40]. This could also affect the male germ line, if mtDNA mutations cause altered mitochondrial function and reduced sperm motility once the affected male germ cells differentiate into spermatozoa [41]. However, due to sperm mitophagy after fertilization, the paternal mtDNA mutations would not be transmitted to offspring.

The mitochondrial bottleneck theory proposes a segregation mechanism of both mutant and wild-type mtDNA. However, the precise explanation of this segregation is still missing. There are three possible mechanisms of the mtDNA bottleneck. First, variation in heteroplasmy is caused by the unequal segregation of mutant and wild-type mtDNA during cell division [37]; second, variation in heteroplasmy is caused by the unequal segregation of homoplasmic nucleoids from multiple mtDNAs during cell division [42]; third, variation in heteroplasmy is caused by the mitochondrial genomes selected for replication [39, 43].

In mice, fertilized oocytes develop into blastocysts by day 4.5 following conception (d.p.c), which is called preimplantation development. The mtDNA copy number remains constant during the preimplantation period [37]. Cree et al. [37] showed that 70% of the heteroplasmy variance was seen at 7.5 d.p.c, which leads to the physical restriction of mtDNA content in early post implantation development of heteroplasmic mice. The remaining 30% were produced during increased proliferation of mtDNA in the expanding germ line. Consequently, Cree and colleagues proposed that the mitochondrial bottleneck is due to the unequal segregation of

mtDNA in early postimplantation development [37]. However, Cao et al. [42] concluded that the mitochondrial bottleneck is not due to the reduction of mtDNA copy number but is caused by the segregation of multiple homoplasmic copies of mtDNA. Despite several hypotheses being proposed, the exact mechanism of the mitochondrial bottleneck is still unknown. In essence, the mitochondrial bottleneck could remove mutations in mtDNA from maternal lineage. This process will then prevent mitochondrial disease in subsequent generations.

2.3. Theories Explaining Clonal mtDNA Inheritance. The fate of paternal mitochondria after fertilization has been a controversial issue in past decades. Early studies presumed that the paternal mitochondria participated in the early embryonic development, while others erroneously thought that the sperm tail with paternal mitochondria was jettisoned before it entered the oocyte at fertilization. As early as 1965, Szollosi [44] reported for the first time that the sperm mitochondria sheath is disassembled by the early eight-cell stage of preimplantation development in the rat embryo.

Although there is no longer doubt that sperm mitochondria enter the oocyte at fertilization, misconceptions about the fate and contribution of paternal mitochondria during preembryo development were still perpetuated in 1980s and 1990s [45]. In early studies, Wilson et al. [46] suggested that vertebrate spermatozoa carry mitochondria and mtDNA in the sperm tail midpiece, but sperm mitochondria do not enter oocyte cytoplasm, or if they do, few survive past one-cell stage [46]. More recently, Lewin incorrectly suggested in a cartoon that the sperm tail including mitochondria is discarded before entering the oocyte at the time of fertilization [47]. Dawkins also reported that mitochondria are inherited from the maternal lineage because the tiny size of a spermatozoon is insufficient to support its own mitochondria [48]. The above hypotheses are not supported with current knowledge showing that sperm mitochondria do enter the mammalian oocyte at fertilization and are actively degraded inside oocyte cytoplasm after fertilization.

Four possible mechanisms should be considered for the lack of paternal mitochondrial gene transmission. The first is a simple dilution effect. A single spermatozoon contains approximately one thousand times less mtDNA molecules than an oocyte [36]. Second, the mitochondrial bottleneck could amplify the dilution of minor paternal alleles during embryonic development [7]. Third, by the time it reaches the oocyte, the fertilizing spermatozoon could contain degraded mtDNA or no mtDNA at all, as recently proposed in *Drosophila* [49] and mouse [50]. Fourth, the active degradation process involving ubiquitination of paternal mitochondria followed by autophagy of the whole mitochondria or proteasomal degradation of extracted mitochondrial proteins acts as a signal for selective elimination inside the oocyte cytoplasm [51]. Evidence for the proteolytic mechanism of sperm mitochondrion elimination has been provided by studies in primate, ungulate, and rodent mammals. More recently, studies linking ubiquitin-proteasome system and autophagy

during sperm mitophagy have been conducted in *Caenorhabditis elegans* [52, 53]. Different species seem to apply various mechanisms to prevent the transmission of paternal mitochondria, as will be discussed below in more detail.

2.4. What Is the Advantage of Clonal mtDNA Inheritance for Individual Fitness and Species Survival? The transmission of mitochondria and mtDNA through the female germ line gives rise to male-female asymmetry, whereas the nuclear but not the mitochondrial genome is contributed equally by both parents. This uniparental inheritance may avoid lethal conflict between genomes [54]. Mitochondria produce ROS that could alter mtDNA integrity; ROS produced during cellular oxidative phosphorylation (OXPHOS) has the potential for causing mutagenic and cytotoxic effects [7]. Therefore, oxidative stress results in a high rate of mtDNA mutation, leading to accumulation of harmful mutated mtDNA known as Muller's Ratchet [55].

Approximately 500 different mtDNA mutations have been associated with degenerative human diseases, cancer, and aging [56–58]. A single pathogenic mtDNA mutant, Leber's hereditary optic neuropathy, is the first known maternally transmitted mitochondrial disorder. Age-related neurodegenerative disorders including Parkinson's and Alzheimer's disease are strongly associated with impaired mitochondrial function [59].

The asymmetric inheritance of mtDNA has an adverse effect on male fitness and male fertility. MtDNA mutations that affect males do not respond to natural selection, though some evidence of sexual selection based on mtDNA sequence was reported in humans [60]. Highly detrimental mutations in mitochondrial genomes are eliminated during female germline development whereas mildly deleterious mutations may be transmitted to the next generation though the female germline [61]. Natural selection will, however, put more pressure on the elimination of female-specific slightly deleterious mutations than male-specific ones because mitochondria are maternally inherited. This selection asymmetry in mtDNA has been described as the mother's curse effect [55]. There is, however, evidence to suggest that specific mtDNA mutations can cause physiological tradeoffs through cellular signalling, which can result in evolutionary advantage in some circumstances [62]. Collectively, these studies illustrate the complexity of mitochondrial inheritance.

Poor male fitness and sperm dysfunction may result from mtDNA mutations that cause a decrease of OXPHOS efficiency. Spermatozoa that possess few mitochondria demand high bioenergetic efficiency for motility, whereas oocytes have low energetic requirements yet possess many times more mitochondria per cell [55]. Therefore, female fertility may be unaffected by a mutation that is detrimental to male fertility. However damaging it may be to male fertility, low levels of deleterious mutated mtDNA will not affect male's fitness until the levels of mutated mtDNA reach a mutation specific threshold level [55, 63].

2.5. Assisted Reproduction and Mitochondrial Fitness. In an effort to alleviate mitochondrial disease, recent research

offers experimental clinical techniques aimed at preventing the transmission of mutant mtDNA [64, 65]. In patients with mitochondrial disease, the mutant mtDNA is either homoplasmic (all mtDNA copies are mutated) or heteroplasmic (both mutant and wild type mtDNA found in the same individual) [66]. The disease phenotypes are only present in patients with above-disease threshold level of mutant mtDNA. One approach to reduce the harmful effect of mutant mtDNA in human embryos created by ART is to transfer the nuclear genome from the zygote with abnormal mitochondria to the recipient zygote/ooplast with healthy mitochondria [64]. Such a pronuclear transfer resulted in normal development to blastocyst, regardless of whether one or two pronuclei were transferred. The pronuclear transfer technique was optimized to minimize the size of the karyoplast with a small amount of cytoplasm, which represents the mtDNA carryover of donor zygote [67]. It remains to be determined what effect, if any, the potential incompatibility between karyoplast nuclear genome and donor cytoplasm mitochondrial genome will have on embryo development. A variation on this technique to prevent transmission of mutant maternal mtDNA is to transplant the metaphase II spindles from unfertilized oocytes containing abnormal mitochondria to those of healthy recipient oocytes, as studied in nonhuman primates [65]. The technique involving the metaphase II spindle transfer has a potential for reducing the level of the carryover mtDNA. Therefore, both the pronuclear and spindle transfer techniques might have the potential for reducing the transmission of mtDNA diseases.

An application of mitochondrial therapy that is particularly relevant to human ART is mitochondrial replacement (MR) therapy. It is well established that oocytes of ART patients of advanced reproductive age (past 35 years of age) have significantly reduced developmental potential, which may be contributed by suboptimal mitochondrial function. Consequently, successful attempts were made early in the century to rejuvenate aged oocytes by infusion of cytoplasm/mitochondria harvested from oocytes provided by donors of prime reproductive age [68]. It was found that this cytoplasmic transplantation generates mtDNA heteroplasmy from donor to offspring in amniocytes, placenta, and fetal cord blood. These attempts were stopped and the ooplasmic transplantation/MT procedure was banned in US and abroad after a report of high incidence of birth defects/developmental anomalies in MR babies [69]. However, the technique has been reintroduced recently in UK, sparking concerns about prenatal development, and postnatal health and fitness of MR children. In particular, numerous examples of detrimental effects of MR/experimentally induced heteroplasmy on health and fitness of MR offspring generated by genetic crossing techniques or by organelle transfer exists in animal models ranging from insects to nonhuman primates [70]. Consequently, some maintain that the reintroduction MR therapy in ART clinics appears premature in absence of extensive animal model testing and safeguarding of human treatments.

3. Maternal Inheritance of mtDNA in Yeast, Nematodes, and Mammals

3.1. Yeast Mitochondria. A yeast cell consists of a single mitochondrial network that exhibits the tubular-reticular structure. Mitochondrial inheritance and dynamics in yeast are maintained by distinct mechanisms: the fission and fusion of mitochondria, the mitochondrial segregation from mother to daughter, and the maintenance of mitochondria [71]. Mitochondrial inheritance is closely synchronized with the cell cycle. Mitochondria align along the mother-bud axis in G1 phase and linear movement of mitochondria from the mother to the bud site occurs during S phase. Mitochondria become immobilized in the bud tip during G2 phase and are eventually released from bud and equally divided between the mother and daughter cell during M phase [72].

Mitochondria in yeast undergo fusion and fission during cell growth, mating, and sporulation. Fusion occurs between the two sides of mitochondrial tubule. Fission occurs along the length of mitochondrial tubules [71]. *Fzo1*, a homologue of the *Drosophila fuzzy onions*, is required for mitochondria fusion in yeast. *Fzo1* protein localizes to the outer mitochondrial membrane. *Fzo1* mutants display mitochondrial fragmentation and loss of mtDNA [73, 74]. The dynamin *Dnm1p*, a GTP-binding protein, is located in mitochondria at division sites. *Dnm1p* is required for mitochondrial fission and essential for normal mitochondrial morphology in yeast. The *Fzo1* and *Dnm1p* double-mutant lack fusion and fission activities. Therefore, normal mitochondrial morphology is controlled by the balance between the expression of *Fzo1* and *Dnm1p* [35, 74].

Mitochondrial transmission from mother to bud progresses along a cytoskeletal track composed of actin patches and actin cables. Mitochondrial movement thus depends on the actin cytoskeleton. The depolarization of actin patches and cables results in the accumulation of mitochondrial particles and loss of mitochondrial movement and abnormal mitochondrial distribution [72, 75]. *Mmm1p*, *Mdm10p*, and *Mdm12p* proteins of the outer mitochondrial membrane are required for segregation of mitochondria to the daughter cell, mtDNA maintenance, and normal mitochondrial morphology [71, 76].

3.2. Nematode Gamete Mitochondria. The nematode *C. elegans* is a self-fertilizing hermaphrodite, having a reproductive organ producing first spermatozoa then oocytes. Self-fertilized embryos proceed through development, hatch, and reach larval stages (L1–L4) [77]. In *C. elegans*, mtDNA inheritance and maintenance for heteroplasmy have been reported [78]. The *uaDf5* mutation is a 3.1 kb deletion that removes a total of 11 genes; it is maternally inherited and has been transmitted for many generations [78]. Because the *uaDf5* mutation is not viable as homoplasmic, it is maintained at ~60% of the mtDNA contents in a stable heteroplasmic condition throughout development. The possible reason for the maintenance of stable heteroplasmy may be that the short, deleted mtDNA molecules have a replicative advantage [79]. Smaller chromosomes could replicate faster over wild-type containing larger chromosomes. However, there is no replicative

advantage in the *uaDf5* mtDNA deletion due to the stable level of *uaDf5* through the development [77].

Observations in *C. elegans* suggest that deletions induced by the removal of the large portion of mitochondrial chromosome result in mtDNA mutation that has severe effects on the carrier's fitness [80]. The experimentally induced mtDNA deletion decreased sperm performance and fitness. It shows that heteroplasmic spermatozoa carrying *uaDf5* deletion of mtDNA crawled more slowly than spermatozoa with wild-type mtDNA, although the sperm fertility was unaffected. The slower rate of egg-laying and shortened life span is also caused by mtDNA *uaDf5* deletion [80].

3.3. Mammalian Sperm Mitochondria. In mammals, mtDNA is typically maternally inherited. Sperm mitochondria are eliminated in the early preimplantation embryo. Although this uniparental inheritance appears to be prevalent in mammals, the underlying developmental mechanisms and timing of sperm mitophagy may vary to some extent among species/taxa. The paternal, sperm borne mitochondria enter the oocyte cytoplasm upon fertilization and are temporally present at the onset of embryonic development. The sperm-contributed mitochondria and mtDNA are degraded and no longer detectable by the time of implantation [45].

Early studies of the elimination of sperm-derived mitochondria in mammals were conducted in mouse, rat, hamster, cow, and pig embryos. Sperm mitochondria are still detected at the two-cell stage in the mouse, and the four-cell stage in the rat and bovine embryo, although some other sperm flagellar structures such as the fibrous sheath disappear before the first embryo cleavage [81]. In the hamster zygote, multivesicular bodies accumulate around the sperm tail and fuse with the mitochondria before their degradation at the two-cell stage [82]. In the bovine embryo, disposal of the sperm mitochondrial sheath is completed in the four-eight cell embryo [83]. Sperm mitochondria in the pig zygote are degraded earlier than the bovine zygote and are undetectable after the first embryo cleavage [84].

Multiple modes of mtDNA inheritance have been described in mammals propagated through interspecies crossing or assisted reproduction. The sperm mtDNA transmission has been observed in various interspecies crosses including sheep interspecies hybrids [85], cloned nonhuman primate [86], and crosses of domestic and wild mice [87]. Kaneda et al. [87] detected paternally inherited mtDNA in *M. musculus* and *M. spretus* interspecies hybrids, whereas sperm mtDNA was eliminated at the early pronucleus stage in one-cell embryos of intraspecific crosses. In this study, interspecific crosses transmitted sperm-contributed mtDNA throughout early embryonic development to the neonate. Additionally, sperm mitochondria from a congenic strain, which derived mtDNA from *M. spretus* and mitochondrial proteins from *M. musculus* nuclear genes, were eliminated by *M. musculus* oocytes presented as intraspecific crosses. This observation suggested that paternal mitochondrial membrane proteins rather than paternal mtDNA itself were recognized by the ooplasmic machinery that seeks and destroys sperm mitochondria. Studies by Gyllenstein et al. [32] also found that sperm mtDNA is present in the first generation

offspring but not inherited in by the subsequent generations of mouse hybrids between *M. musculus* C57BL/6J strain and *M. spretus*. It can be concluded that the elimination of sperm mtDNA is a species-specific mechanism that is circumnavigated by mouse interspecific hybrids.

Somatic cell nuclear transfer (SCNT) is inherently conducive to heteroplasmy because donor somatic cell mitochondria likely lack the protein marks that would make them recognizable to the ooplast as being foreign mitochondria. Besides the mismatch between donor and recipient mitochondrial genomes, abnormal interactions of mitochondrial proteins encoded by mtDNA from a recipient oocyte with a donor cell's mitochondrial proteins encoded by donor nuclear DNA could contribute to reduced fitness of embryos reconstructed by SCNT. In previous studies of bovine [88], murine [89], and porcine [90] SCNT offspring, heteroplasmy derived from the transmission of donor cell mtDNA was observed at varied ratios of donor-to-recipient mtDNA. A relatively small level of donor cell mtDNA was detected in the naturally conceived offspring of cloned pigs, which is in accordance with natural segregation of donor mtDNA leading to the concept of a genetic bottleneck in cloned germ line [90]. The ratio of donor mtDNA in cloned offspring reveals tissue-specific distribution in mouse brain, liver, and tail [89]. Interspecies somatic cell nuclear transfer (iSCNT) has been applied to various species, including gaur/bovine [91]. In gaur/bovine iSCNT embryos, the donor mtDNA from fibroblast was detectable at all stages of preimplantation development with varying degree of heteroplasmy among individual cloned embryos [91]. High levels of mtDNA heteroplasmy might contribute to lesser efficiency of iSCNT compared to intraspecific SCNT.

4. Sperm Mitophagy in the Mammalian Zygote Is Mediated by Ubiquitin-Proteasome System

How could sperm-borne mitochondria be selectively degraded after fertilization in mammals without the mitophagy mechanism attacking the oocyte's own mitochondria? Early work in bovine, rhesus monkey, and murine zygotes showed that once they enter the oocyte cytoplasm, the sperm mitochondria are tagged with ubiquitin, which is thought to flag the proteins and possibly organelles for degradation by the ubiquitin-proteasome system (UPS) [92]. In bovine *in vitro* embryo culture, paternal mitochondria are eliminated between 4- and 8-cell stages. Interestingly, sperm mitochondria in interspecific bovine cross embryos were not ubiquitinated and still present at the eight-cell stage [92]. Such an observation agrees with the persistence of paternal mtDNA in the interspecific mouse crosses [93], suggesting that the mechanism for selective sperm mitophagy has a species-specific element.

The UPS is an essential, tightly regulated route for substrate-specific protein degradation in eukaryotes. Consequently, UPS is a good candidate for selective degradation of paternal mitochondrial proteins after fertilization [94]. Ubiquitin is a highly conserved small proproteolytic chaperone

protein present in the cell cytoplasm and nucleus and, in some instances, in the extracellular space and on the cell surface [95]. Protein ubiquitination signifies the covalent ligation of one or more ubiquitin molecules to substrate proteins through a sequential action of at least three enzyme activities: a ubiquitin-activating enzyme E1, a ubiquitin-conjugating enzyme E2, and a substrate-specific ubiquitin ligase E3 [96]. Upon the ligation of one ubiquitin molecule to substrate's internal Lys residue (monoubiquitination), additional ubiquitin molecules can attach to one of the Lys-residues of that substrate-bound monoubiquitin in a tandem fashion, again through linkage to one of its seven internal Lys-residues (K6, K11, K27, K29, K33, K48, and K63), to form multiubiquitin chains [97].

Protein tagging by polyubiquitin chains culminates in recognition and proteolysis by the 26S proteasome, a multi-subunit protease holoenzyme. The 26S proteasome is typically composed of a 20S proteasomal core particle, a complex of four concentric rings forming a hollow barrel-shaped structure, and a cap-like structure of the 19S proteasomal regulatory complexes on one or both ends of the 20S core. The 19S complex recognizes, engages, and removes the multiubiquitin chain on the ubiquitinated substrate protein, which is then primed and translocated to the 20S core for degradation by three resident proteases (20S core subunits PSMB5, PSMB6, and PSMB7). The substrate protein is broken down into small peptides of 3–20 amino acids, released from the proteasome and degraded to individual amino acids by cytosolic endopeptidases [97, 98].

During mammalian spermatogenesis, mitochondrial ubiquitination is detected at the secondary spermatocyte phase, followed by the round spermatid, and finally in fully differentiated testicular spermatozoa of the bull [94]. After incorporation into the oocyte cytoplasm during fertilization, ubiquitinated sperm mitochondria are detectable in the oocyte cytoplasm whereupon the intensity of ubiquitin binding appears to increase, as detected by immunofluorescence imaging [51]. In porcine zygotes, the degradation of the sperm mitochondrial sheath was delayed by the specific, reversible proteasomal inhibitor MG132 and resumed once the inhibitor was washed off [84]. Paternal mitochondria in porcine zygotes appear to be degraded prior to the first embryo cleavage following *in vitro* fertilization. Lactacystin, an irreversible proteasomal inhibitor, also prevents sperm mitophagy in the porcine zygote. Such data indicate that the elimination of ubiquitinated proteins can be controlled by proteasomal activity, which, as we will discuss below, is linked to autophagic pathway for organelle degradation. The proteasomal inhibitors also block the penetration of the mammalian egg coat, zona pellucida (ZP), which suggests the role of sperm proteasome during the sperm-ZP interactions [84]. Consequently, proteasomal inhibitors had to be added to the fertilization medium after sperm-zona penetration was completed, in order to assess their effect on sperm mitophagy.

5. The Role of Autophagic Pathway in the Sperm Mitochondrion Degradation

Following an early indication of lysosomes contributing to sperm mitophagy in the bovine embryo [94], recent studies in *C. elegans* support the hypothesis that the ubiquitin and lysosome-dependent autophagic pathway actively participate in sperm mitophagy after fertilization. Autophagy, from the Greek word meaning “to eat oneself,” is conserved in all eukaryotes. Various macromolecules and whole organelles are delivered from the cytoplasm to lysosomes for degradation. In the process of autophagy, protein-aggregates, defective cellular structures, and damaged organelles are engulfed by double-membrane vesicles called autophagosomes. These vesicles with cargo destined for degradation are targeted to the lysosome, the major digestive organelle in the cell. After approaching the lysosomes, autophagosome-lysosome fusion occurs to form autolysosomes. Eventually, degradation takes place through the mediation of lysosomal hydrolase enzymes [99].

Three recent studies in *C. elegans* implicate lysosome and autophagy processes in the degradation of paternal mitochondria in the early embryo [52, 53, 100]. The degradation of sperm borne mitochondria is associated with the lysosome that is a digestive organelle containing hydrolytic enzymes. Treatment with NH_4Cl , a lysosome inhibitor, delays paternal mitochondrial elimination after fertilization. The participation of lysosome pathway implicates the involvement of autophagy pathway as an upstream process of the lysosomal degradation [100]. The *C. elegans* sperm mitochondria are near the ER/Golgi derived membranous organelle (MO), which is essential for sperm motility. Fertilization in *C. elegans* triggers a selective autophagic response. Sperm mitochondria and MOs in *C. elegans* enter the oocytes upon fertilization, and the paternal mitochondria disappear by the 16-cell stage of the embryonic development [52, 53]. Autophagosomal membrane proteins LGG-1 and LGG-2 (homologs of mammalian GABARAP and LC3, resp., Table 1) accumulate around both paternal mitochondria and MOs in the oocyte cytoplasm. Furthermore, paternal mitochondria persist in the late stage embryos past 16 cells in the LGG-1 knockout worms. It appears that the sperm-derived mitochondria are engulfed by the autophagosomes and eliminated by autophagic pathway in the nematode embryo. Notably, sperm-derived MOs are tagged with ubiquitin in the oocyte cytoplasm and are subsequently recognized by the autophagosome for autophagic degradation [52, 53]. Although paternal mitochondria are tagged with ubiquitin during spermatogenesis and after fertilization in mammals, paternal mitochondria do not appear to be ubiquitinated in *C. elegans*.

Interestingly, the comparative experiments in *C. elegans* [52, 53] suggest that sperm mitochondrion autophagy is also conserved in mammals. The autophagy related proteins, such as LC3, GABARAP, and p62 (Table 1), were detected around the midpiece of the fertilizing spermatozoa inside mouse embryos. Autophagy is triggered when mouse spermatozoa enter the oocyte after fertilization and may have functions other than sperm mitophagy since it appears to be required

TABLE 1: Nomenclature and aliases of autophagy-related genes.

Full name (human)	Yeast	<i>C. elegans</i>	Human/mammalian	Aliases	Detectable in spermatozoa	Function
Autophagy-related 5	N/A	Atg5	ATG5	ASP; APG5; APG5L; hAPG5; APG5-like	No	Early stage of autophagosome formation
GABA(A) receptor-associated protein	ATG8	Lgg-1	GABARAP	MM46; ATG8A; GABARAP-a	Yes	Nonselective sequestration of cytoplasmic material for vacuolar degradation
Microtubule-associated protein 1 light chain alpha	N/A	Lgg-2	MAP1LC3A	LC3; LC3 A; ATG8E; MAP1ALC3B	Yes	Recruits protein cargo to the phagophore/isolation membrane; remains associated with the mature autophagosome
Sequestosome 1	N/A	N/A	SQSTM1	p62; p60; A170	Yes	Binds to ubiquitinated proteins; interacts with both LC3 and GABARAP
Valosin containing protein/protein dislocase	CDC48	CDC-48.1	VCP	p97	Yes	Extracts ubiquitinated proteins from organelle membranes; presents them to 26S proteasome
Histone deacetylase 6	N/A	HAD-6	HDAC6	HD6	Yes	Transports ubiquitinated misfolded protein aggregates/aggresomes to phagophore
Parkin (PARK2)	N/A	pdr-1	PARK2	PDJ; PRKN	Not known	Ubiquitin ligase; induces selective autophagy of damaged mitochondria

for mouse embryo survival; the *Atg5*^{-/-} mouse embryos die by the eight-cell stage [101].

The accumulation of autophagosomal markers in the vicinity of ubiquitinated sperm mitochondria in *C. elegans* and mouse embryos reflects evolutionary conservation of the proteolytic mechanism for the elimination of paternal mitochondria after fertilization. Such findings also hint at the synergy between autophagy and UPS during sperm mitophagy inside the fertilized oocyte/zygote.

Recent study of mouse embryos challenged the role of autophagic pathway in murine mitochondrial inheritance [50]. Oocytes of transgenic female mice expressing the green fluorescent protein (GFP) tagged autophagosome protein LC3 were fertilized with spermatozoa of a transgenic strain expressing red fluorescent protein (RFP) in mitochondria. Authors found that red fluorescent structures purported to be sperm mitochondria remained detectable up to morula stage but the RFP fluorescence did not colocalize with GFP-LC3 or with LysoTracker labeled lysosomes, leading them to conclude that autophagic pathway is not required for the elimination of paternal mitochondria after fertilization. Lack of LC3 association with sperm mitochondria beyond early stages of embryo development agrees with our preliminary studies in porcine model (Figure 2) but by no means allows concluding that autophagy is not involved, since other branches of the autophagic pathway need to be considered. These authors also reportedly detected paternal mtDNA in a small proportion of mouse pups born after intraspecific crossing. As an alternative to postfertilization mitophagy, Luo et al. suggested that most mouse spermatozoa that reach the site of fertilization in the mouse oviduct lack mtDNA

and speculated that male germ line mtDNA is degraded during spermiogenesis [50]. While intriguing, the proposed mechanism of passive elimination of paternal mtDNA fails short of explaining high incidence of paternal heteroplasmy in mouse interspecific crosses reported previously [32, 87]. It is also unclear whether red fluorescent structures detectable beyond the four-cell stage are intact sperm mitochondria or just remnant of mitochondrial membranes, and why it would be possible to detect paternal mtDNA in offspring if the sperm-mitochondrion derived RFP fluorescence was no longer detectable at blastocyst stage. Such data will likely be scrutinized from the point of view of sensitivity and specificity of paternal mtDNA detection in single spermatozoa, embryos, and offspring, as was the case recently in *Drosophila* [49, 102].

6. The Crosstalk between Autophagy and Ubiquitin-Proteasome System during Sperm Mitophagy

6.1. Possible Scenarios for the Synergy between Ubiquitin-Proteasome System and Autophagy during Sperm Mitophagy. Autophagy and the UPS are the two major cellular protein degradation pathways. Recent work has made it increasingly clear that ubiquitin-binding proteins/receptors in autophagy mediate interplay between the two systems. Hypothetically, at least three well characterized pathways involving autophagy and UPS could act in synergy during sperm mitophagy: (1) autophagy-associated ubiquitin-receptor p62/SQSTM1 recognizes ubiquitinated cargo and

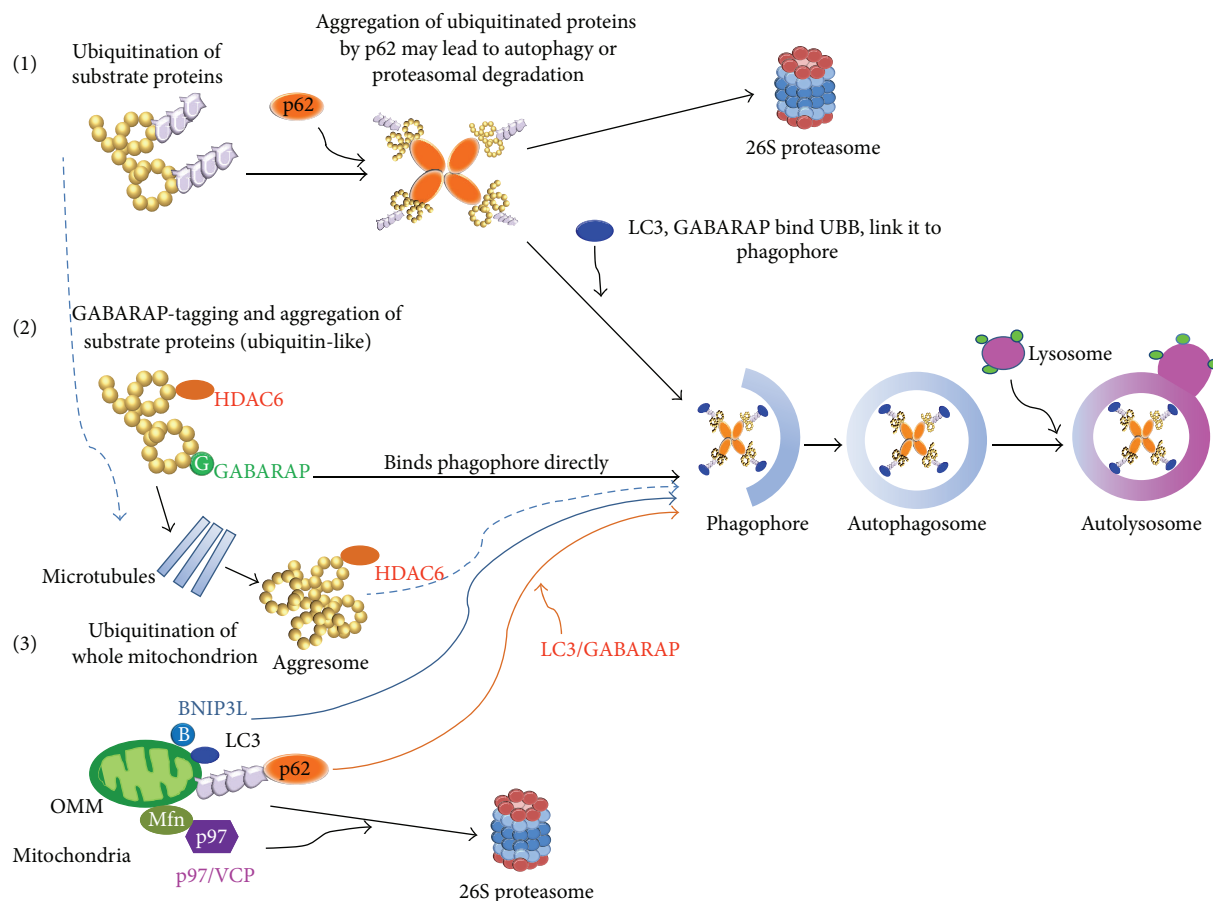


FIGURE 1: Diagram of candidate pathways leading to sperm mitophagy by autophagy and ubiquitin-proteasome system. Generally, the process of autophagy starts with the aggregation and ubiquitination of proteins or organelles that need to be recycled. Multiubiquitin chains on such aggregates are recognized by the ubiquitin-binding autophagy receptors and are brought to phagophore, a membranous organelle that eventually closes around the protein aggregate to form an autophagosome. In the final step, autophagosome fuses with a lysosome that contains proteases able to degrade the protein cargo. In some branches of this pathway, protein aggregates or ubiquitinated proteins extracted from organelle membranes are targeted for degradation by the 26S proteasome, a multisubunit ubiquitin-specific protease. At least three previously characterized pathways could be involved in the degradation of sperm mitochondria inside a fertilized oocyte: (1) Autophagy-associated ubiquitin-receptor p62/SQSTM1 recognizes ubiquitinated cargo and interacts with autophagosome-binding ubiquitin-like proteins, such as LC3 or GABARAP; these autophagy receptors guide the protein cargo to phagophore; (2) ubiquitinated proteins of mitochondrial origin form aggresomes, the protein aggregates induced by the ubiquitin-binding adaptor protein HDAC6, which transport the ubiquitinated proteins towards degradation site, the phagophore, along microtubule tracks. (3) Protein dislocase p97/VCP extracts and presents the ubiquitinated mitochondrial membrane proteins to the 26S proteasome, the ubiquitin-dependent protease, without the involvement of phagophore.

interacts with autophagosome-binding ubiquitin-like modifiers, such as LC3 or GABARAP; (2) ubiquitinated mitochondria or mitochondrial proteins may form aggresomes, the protein aggregates induced by the ubiquitin-binding adaptor protein HDAC6, which transport such ubiquitinated protein aggregates along microtubules; and (3) the protein dislocase p97/VCP has the ability to extract and present ubiquitinated mitochondrial membrane proteins to the 26S proteasome, the ubiquitin-dependent protease. All three pathways may converge at the time of the formation of autophagic vacuole, as shown in Figure 1. According to this scenario, the process of sperm mitophagy starts when the ubiquitinated sperm mitochondria or mitochondrial protein aggregates dock to preautophagic membrane of the phagophore, then become

engulfed in autophagosome, and fuse with lysosome to complete organelle/aggregate proteolysis. Alternatively, some branches of the three pathways proposed above, and most notably the p97/VCP dependent pathway, could channel the dislocated ubiquitinated mitochondrial membrane proteins directly to the 26S proteasome. It should also be considered that UPS could regulate the autophagic branch of these pathways indirectly by controlling the turnover of autophagic ubiquitin receptor proteins.

6.2. Ubiquitin-Binding Proteins Serve as Autophagy Receptors.

In the last decade, genetic screens identified approximately 30 autophagy-related (Atg) proteins in yeast that mediate autophagosome formation and cargo degradation in

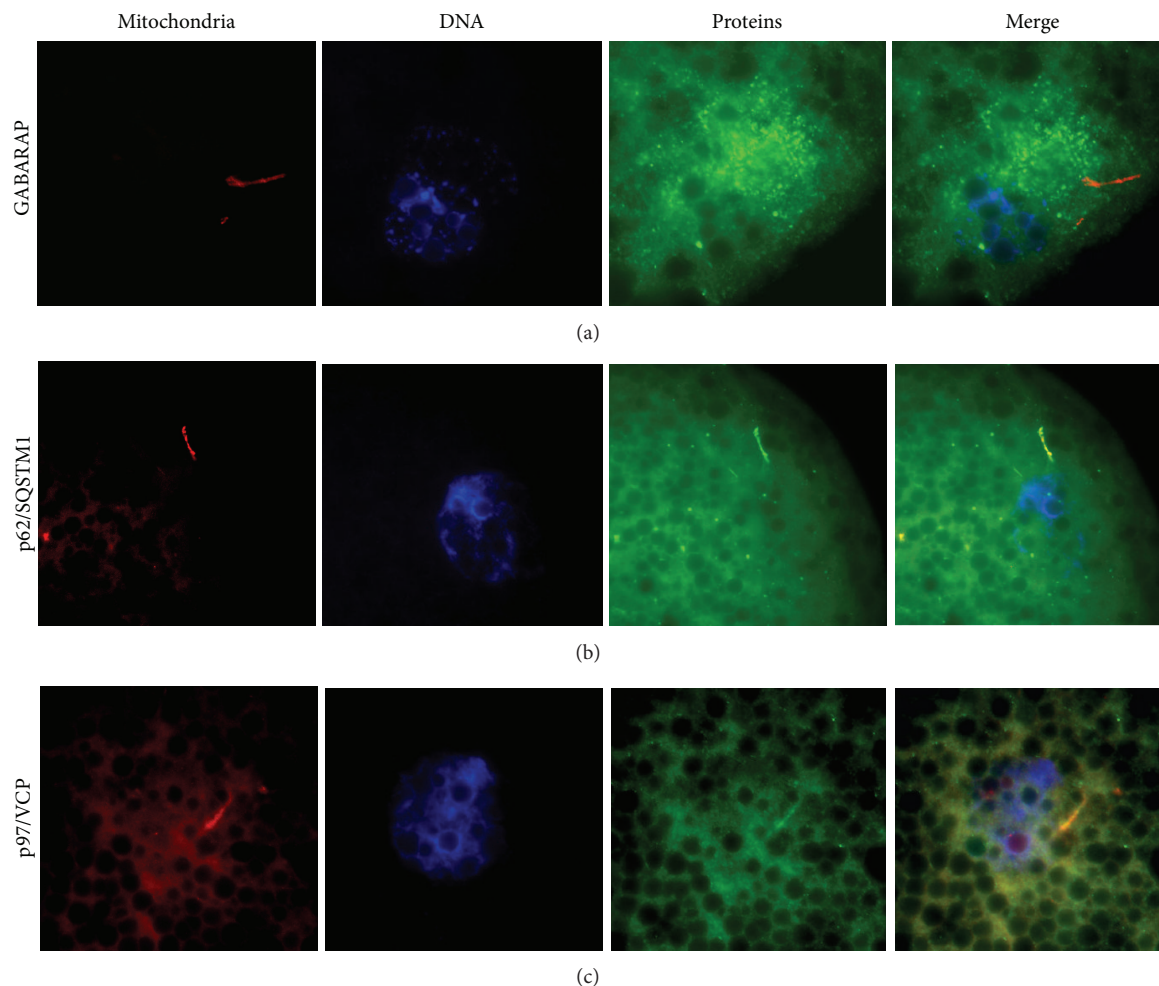


FIGURE 2: Immunofluorescence localization of GABARAP, p62/SQSTM1, and VCP in porcine zygotes. (a) Autophagy receptor/ubiquitin-like protein GABARAP accumulates around male pronucleus and sperm mitochondria of an embryo treated with proteasomal inhibitor MG132 (100 μ M) at 30 hr after insemination. Inhibition of proteasomal proteolysis is known to induce compensatory activation of autophagic pathway. (b) Ubiquitin-binding protein p62/SQSTM1 is detected in the mitochondria region of spermatozoa in embryo cultured for 30 hr after IVF. SQSTM1 is an ubiquitin-receptor that links UPS to autophagic pathway. (c) Protein dislocase p97/VCP is present in the mitochondrial sheath of spermatozoa in a porcine zygote cultured for 30 hr after IVF. Dislocase VCP recognizes and extracts ubiquitinated mitochondrial membrane proteins, presenting them to 26S proteasome for degradation.

the autophagic body [103] (Table 1). Mammalian LC3 and GABARAP are located in the phagophore membrane and help attach ubiquitinated proteins to the autophagosome [104]. Both LC3 and GABARAP are members of the ubiquitin superfamily and facilitate selective degradation of ubiquitinated proteins [105]. The LC3 binds directly to the p62/SQSTM1 protein, which is found in inclusion bodies containing ubiquitinated protein aggregates and has a C-terminal ubiquitin-associated (UBA) domain that engages ubiquitinated proteins [106]. The SQSTM1 also contains a PB1 domain to facilitate self-oligomerization. A specific region of SQSTM1, known as the LC3 recognition sequence (LRS), is formed by the Asp337-Asp339 acidic cluster [106, 107]. The N-terminal α -helix surface of LC3 interacts with LRS and/or LIR (LC3-interacting region) of UBA domain.

The SQSTM1-derived UBA domain can bind both Lys 48-linked and Lys 63-linked multiubiquitin chains [108].

Growing evidence suggests that the affinity of SQSTM1 to ubiquitinated proteins results in their subsequent transport to the autophagosome for degradation [109]. The SQSTM1, an autophagic adaptor, accumulates in damaged mitochondria and contributes to autophagic degradation [110]. The SQSTM1-deficiency inhibits the accumulation of LC-positive autophagosomes during amino acid starvation [109]. These findings strongly indicate that the ubiquitinated protein cargo is recognized by SQSTM1 and interacts with LC3 and/or GABARAP on the phagophore membrane. Preliminary evidence from our porcine mitophagy model [111] and comparative data from *C. elegans* and mouse [53] suggests

that paternal mitochondria in mammals are degraded with the help of zygotic, oocyte-derived autophagy-associated ubiquitin-receptors.

6.3. Histone Deacetylase HDAC6 Links Transport Ubiquitinated Cargo along the Microtubule Cytoskeleton. The crosstalk between the autophagic pathway and the UPS may also utilize the histone deacetylase 6 (HDAC6), which has the ability to bind to misfolded ubiquitinated proteins on one side and to dynein-binding cytoskeletal adaptor proteins on the other [112]. The HDAC6 has an ubiquitin-binding zinc-finger, called BUZ domain, which facilitates ubiquitin-binding to HDAC6 and is necessary for aggresome formation and degradation [113]. HDAC6 does not possess a LIR motif but rather interacts directly with dynein motors, which transport the aggregated, misfolded proteins along microtubules to the microtubule-organizing center (MTOC) where aggresomes tend to accumulate. In mammalian fertilization, the zygotic centrosome organizes a microtubule sperm aster and is close to the sperm mitochondria since it is derived from the sperm-borne centriole [114]. Notably, failure of human assisted fertilization is associated with protein aggregation and proteasome accumulation around the sperm-derived zygotic centrosome [115]. Although the aggregated ubiquitinated proteins, also known as aggresomes, are not eliminated primarily by proteasomal proteolysis, they contain proteasomes; aggresome formation typically signals an increased need for protein recycling and sometimes indicates the saturation of UPS with undegraded ubiquitinated proteins, associated with cellular stress. HDAC6-deficient cells fail to concentrate misfolded proteins into aggresomes and will instead retain them as cytoplasmic aggregates [116]. These observations suggest that HDAC6 is a crucial mediator of aggresome formation and a candidate participant in zygotic sperm mitophagy.

Aggresomes are localized around the MTOC, and aggresome formation requires the microtubule network to transport polyubiquitinated misfolded proteins. Dysfunction of the MTOC or the dynein motor leads to disruption of aggresome formation [117]. This observation indicates that the HDAC6-engaged polyubiquitinated proteins bind to dynein motors and move toward the MTOC, delivering the aggresome for degradation. It has been proposed that the bulk-delivery of misfolded proteins in the form of aggresomes facilitates proteolysis via autophagic route, ending with fusion of the autophagosome and lysosome [116]. When the UPS is impaired, autophagy is accelerated to compensate for UPS dysfunction in HDAC6-dependent manner [118]. Importantly, recent studies suggest that HDAC6 mediates the clearance of damaged mitochondria. Ubiquitin ligase, Parkin, induces the ubiquitination of impaired/damaged mitochondria, leading to the recruitment of HDAC6. The target mitochondria are then cleared by HDAC6-dependent autophagy [119]. It can therefore be speculated that the sperm mitochondria could also be degraded by HDAC6 mediated selective autophagy.

Considering that a 26S proteasome can only degrade one protein molecule at a time (as opposed to a whole organelle or

organelle membranes), it is prudent to ask how proteasomes could degrade a whole sperm mitochondrion inside a fertilized mammalian oocyte. To overcome this seeming paradox, one can focus on the ability of certain ubiquitin-binding protein dislocases to extract ubiquitinated proteins from the outer mitochondrial membrane and deliver them to the 26S proteasome. Best characterized among these “substrate-presenting,” proteasome-binding proteins are the valosin containing protein VCP (alias p97).

6.4. Protein Dislocase VCP Extracts Ubiquitinated Proteins from the Mitochondrial Membrane and Presents Them for Degradation. The vertebrate VCP (called Cdc48 in yeast), is a member of AAA-ATPase family involved in many cellular processes such as cell division, endoplasmic reticulum-associated protein quality control (ER-associated degradation/ERAD), and ubiquitin-dependent proteolysis. Recent studies also implicate VCP in regulating autophagy-mediated protein degradation via ubiquitin-dependent process. The VCP dislocase serves as the “motor” that mediates those cellular functions by binding to specific cofactors including Ufd1, Npl4, and p47. Those cofactors have ubiquitin-binding domains and interact with VCP as ubiquitin adaptors [120–122]. Based on the general model of VCP function within UPS, the target substrate is ubiquitinated by E1-E2-E3 enzyme cascade and VCP engages the polyubiquitin tail on the substrate protein through the above cofactors. The VCP then uses the energy from ATP hydrolysis to extract the ubiquitinated protein by separating it from its binding partners on the organelle membrane, and presenting it to the 26S proteasome for recycling [122]. Alternatively, the VCP-dislocated proteins can be delivered for autophagic protein degradation by VCP-mediated autophagosome. The importance of VCP for autophagy is confirmed by the inclusion body/familial VCP myopathy associated with frontotemporal dementia and Paget’s disease of bone, caused by mutations in VCP [123]. The loss of VCP activity impairs maturation of ubiquitin-containing autophagosomes, which consequently fail to promote autophagy [124, 125].

The specific role of VCP during somatic cell mitophagy is to extract ubiquitinated outer mitochondrial membrane (OMM) proteins to the cytosol, for proteasomal degradation. The degradation of OMM-associated proteins, such as Mfn1 and Mfn2, is mediated by 26S proteasome in VCP-dependent manner [126, 127]. The OMM contains or can accommodate several E3 ubiquitin ligases. Specifically, Parkin, an E3 ubiquitin-ligase, is recruited to mitochondria and participates in mitophagy [127–129]. The mutation of Parkin-encoding *Park2* gene causes Parkinson’s disease, which is associated with mitochondrial defects. Parkin mediates the elimination of the defective mitochondria by autophagosomes [129]. In addition, Parkin initiates the ubiquitination of OMM proteins, MFN1 and MFN2, thereby inducing both proteasome-dependent degradation and VCP-dependent mitophagy [129, 130]. Interestingly, *Drosophila* males lacking the Parkin gene are sterile and display mitochondrial pathology associated with the failure of spermatid individualization late in spermatogenesis [131]. Altogether, Parkin-mediated

ubiquitination and VCP-induced degradation of ubiquitinated proteins can promote mitochondrial degradation by a dual route involving autophagy/mitophagy and proteasome-dependent degradation.

7. Conclusions

The mitochondria exist in almost all eukaryotic cells and are important for cellular energy production, calcium signaling, apoptosis, and many other cellular functions. Maternal inheritance of mitochondria and their DNA is universally observed in humans and most animals. The mutation and/or transmission of paternal mitochondrial genome are associated with various human diseases. The elimination of paternal mitochondria shortly after fertilization is the first line of defense to prevent potentially dangerous mitochondrial-genomic dysfunction. Reviewing current literature on mitochondrial inheritance, the elimination of paternal mtDNA can be accomplished by multiple mechanisms. Ubiquitination of germ cell mitochondria is observed during mammalian spermatogenesis and also detected after fertilization. The ubiquitinated sperm mitochondria typically disappear from early stage preimplantation embryos, while the exact timing of sperm mitophagy appears to vary among mammalian species. Embryo treatments with proteasomal and lysosomal inhibitors indicate the existence of a two-way mechanism of sperm mitochondrion degradation involving both proteasomal and lysosomal proteolysis, the latter being an autophagy/mitophagy endpoint.

The recent findings in *C. elegans* help explain how ubiquitin-proteasome system and autophagy cooperate during the degradation of paternal mitochondria and mtDNA by the early embryo. Autophagy-related ubiquitin-receptors are detected in the paternal mitochondria inside the fertilized oocyte. It is thus possible that specific autophagy-related ubiquitin-binding proteins such as GABRAP, LC3, HDAC6, VCP, and SQSTM1 promote uniparental inheritance of mitochondria in mammals, as suggested by our preliminary data (Figure 2). Most likely, the interplay between proteasome-dependent degradation and sperm mitophagy exists in mammals and other taxa. Work is in progress to identify the mechanistic links between UPS and sperm mitophagy, which could impact the development of new treatments for human mitochondrial disease and infertility.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Power of Proteomics in Linking Oxidative Stress and Female Infertility

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Endometriosis, PCOS, and unexplained infertility are currently the most common diseases rendering large numbers of women infertile worldwide. Oxidative stress, due to its deleterious effects on proteins and nucleic acids, is postulated to be the one of the important mechanistic pathways in differential expression of proteins and in these diseases. The emerging field of proteomics has allowed identification of proteins involved in cell cycle, as antioxidants, extracellular matrix (ECM), cytoskeleton, and their linkage to oxidative stress in female infertility related diseases. The aim of this paper is to assess the association of oxidative stress and protein expression in the reproductive microenvironments such as endometrial fluid, peritoneal fluid, and follicular fluid, as well as reproductive tissues and serum. The review also highlights the literature that proposes the use of the fertility related proteins as potential biomarkers for noninvasive and early diagnosis of the aforementioned diseases rather than utilizing the more invasive methods used currently. The review will highlight the power of proteomic profiles identified in infertility related disease conditions and their linkage with underlying oxidative stress. The power of proteomics will be reviewed with regard to eliciting molecular mechanisms for early detection and management of these infertility related conditions.

1. Introduction

Current studies estimate that 17% of couples are confronted with an inability to conceive and their infertility could stem from different causes, either affecting the male or the female. Around 50% of these are due to the female factor and associated diseases in the female reproductive tract [1]. When defining infertility, it has been noted that couples are termed infertile if they are unable to conceive after 12 months of unprotected and regularly timed sexual intercourse [2]. The desire to procreate is naturally present in women worldwide. Due to various advances in medical techniques it has now become possible for many women to fulfill the hope of mothering children of their own. The longing to conceive a child has been recently proposed to be one of the risk factors for women's depression. Furthermore, the added financial burden is an impediment to economic status as these women are more likely to end up paying for assisted reproductive techniques (ART). Several medical advances during the past decade and a half have led to the establishment of ART clinics worldwide that provide services to infertile couples to help

them achieve pregnancy. Chachamovich et al. showed that women were more likely to be psychologically distressed due to infertility than men and that they suffer from poor quality of life once they are diagnosed as infertile [3].

There seems to be multiple reasons for female infertility, of which the most common are endometriosis related infertility, ovulatory disorders, tubal factor infertility, and unexplained infertility. Women suffering from any of the above-said diseases have been shown to have a lower chance of conceiving a child and are more likely to seek infertility treatment [4].

Several studies have linked the prevalence of female infertility with an increase in oxidative stress levels in the various critical micro- or macroenvironments in the body. Oxidative stress results from an increase in reactive oxygen species (ROS), as the antioxidant capacity of the cells to scavenge and remove these free radicals decreases. ROS is the byproduct of the process of respiration taking place in the mitochondria [5]. Commonly, ROS molecules interact with proteins, lipids, carbohydrates, or DNA molecules within cells, causing damage to cellular structures including cell membranes and

genetic material [6]. The imbalance of ROS can eventually lead to epigenetic differences and changes in cellular pathways and transcription factors [7]. Studies have shown that oxidative stress causes alterations in certain protein pathways and the abnormal expression of several proteins could possibly lead to the pathophysiology of female infertility. The abnormally expressed proteins could be integral factors responsible for oxidative stress reaction, or antioxidants or different binding proteins that may bind oxidants and antioxidants such as superoxide dismutase (SOD), paraoxonase (PON), hemopexin, apolipoproteins, and heat shock proteins [8–13].

The study of the expression of different proteins in a cell or tissue in a temporal and spatial fashion is referred to as proteomics [14]. This technique is fairly new and has been advancing at an extraordinary pace, as new methodologies evolve for the detection of minute amounts of proteins in different body fluids. Since proteins dictate cellular functions to a large extent, comparative proteomics investigating various proteins in the normal and diseased samples is deemed to be an important factor in the diagnosis and treatment of diseases [15]. Comparative proteomic analyses could possibly aid in the identification of biomarkers for noninvasive diagnosis of female diseases and assist in the prediction of success rates for the assisted reproduction techniques (ART).

The purpose of this review is to examine the relationship between oxidative stress and differentially expressed proteins in female infertility that has been identified by proteomic techniques. The review will focus on the proteins involved in endometriosis, polycystic ovarian syndrome, and unexplained infertility and those involved in examining the embryonic secretome associated with ART. Finally, our review will illustrate the power of proteomics in the discovery of biomarkers for noninvasive diagnosis as well as prediction of ART success rate.

2. Oxidative Stress and Female Fertility

Reactive oxygen species (ROS) are a class of free radicals lacking one or more electrons in their outer shell. They are highly reactive and have a tendency to interact with neighboring molecules to stabilize their structure. The electron transport chain in the mitochondria is an important endogenous source of ROS. Several exogenous factors, namely alcohol, smoking, and environmental factors, can enhance the ROS production [16]. The free radicals commonly associated with oxidative stress include hydroxyl radicals, superoxide anion, and hydrogen peroxide [17].

Enzymatic antioxidants such as glutathione oxidase and superoxide dismutase as well as nonenzymatic antioxidants (vitamin A, vitamin E, zinc, and selenium) are essential in maintaining adequate levels of ROS in the cell by disposing and removing excess free radicals [7]. Any disruption in the antioxidant/ROS balance leads to a state of oxidative stress in the cell with damaging consequences.

Physiological levels of ROS are required for proper functioning of different biological pathways and in maintaining homeostasis within the human body. Low levels of free

radicals act as modulators in female reproductive pathways such as oocyte maturation, physiological follicular atresia, ovulation fertilization, luteal regression, and corpus luteum formation during pregnancy [18]. At puberty, monthly maturation of a primordial follicle into a graafian follicle occurs. Normal levels of ROS help in the resumption of meiosis-I and also in the development of a dominant oocyte, and increased concentrations of antioxidants have been shown to inhibit this process. Conversely, antioxidants promote resumption of meiosis-II [19] allowing for fertilization at the metaphase II level. Therefore, follicle maturation is a classic example of the delicate balance that exists between ROS and antioxidants in the maintenance of the regulated sequence of events that culminate in ovulation. As the follicle grows, hormonal secretions increase and result in elevation of Cytochrome P450. Cytochrome P450 enzymes can undergo changes in the absence of a substrate to produce ROS including superoxide anions, hydroxyl radicals, and hydrogen peroxide. CYP 2E1, one of the enzymes in the P450 complex, is thought to induce NADPH-dependent lipid peroxidation. Another component of the P450 complex, CYP4A, is associated with the production of superoxide and hydrogen peroxide [20]. The increase in ROS in pre-ovulatory follicle is crucial for the onset of ovulation [5].

ROS is also believed to play a role in the different phases of the endometrial cycle. Late luteal phase is characterized by elevated levels of lipid peroxide and a decrease in the antioxidant, superoxide dismutase. ROS stimulates the secretion of PG2F α through activation of NF κ B (nuclear factor-kappaB) [21]. Decreased levels of estrogen and progesterone lead to a decreased SOD expression and hence generate oxidative stress in the uterus, resulting ultimately in endometrial shedding and lack of implantation. Controlled levels of ROS have, however, been associated with the angiogenic activity in the endometrium causing regeneration in every cycle. These studies show that limited levels of ROS are necessary to maintain physiological function, but when present in higher concentrations, ROS can have deleterious effects [22].

Disruption in physiological levels of ROS leads to female reproductive dysfunction and in some cases, to unexplained infertility. Oxidative stress in female reproduction has been associated with polycystic ovarian syndrome and endometriosis. Needless to say, these pathologies negatively affect pregnancy rates and IVF outcomes.

ROS is produced at multiple sites in a preovulatory follicle and renders it, a susceptible medium for oxidative stress. ROS levels can be measured in several different media including the follicular fluid (FF), endometrial fluid, and peritoneal fluid. The FF contains ROS producing agents, including cytokines, neutrophils, and macrophages. Steroidogenesis involves a series of enzymatic reactions which result in the formation of steroid hormones. The monooxygenase pathway is mediated by p450 and results in production of ROS [23] rendering the FF as a suitable medium for oxidative stress measurement.

Even though abnormal increase in ROS levels in women is negatively correlated with (1) oocyte development [24], (2) embryonic development [25], and (3) pregnancy outcome [26], physiological levels of ROS are required for healthy

development of oocytes and better IVF outcomes [27]. This makes it important to determine the threshold value of ROS, beyond which its negative effects in fertility are observed. Jana et al. determined the upper cut-off value of ~100–107 counted photons per sec (cps) of ROS in follicular fluid, above which ROS negatively affects fertility and IVF results. In women with tubal factor infertility, undergoing IVF, ROS cut-off levels were determined to be 100 counted photons per second (cps). With ROS levels lower than 100 cps, embryo formation and embryo quality were enhanced compared to those with ROS levels above 100 cps [28]. When the same study was performed on patients with PCOS and endometriosis, the upper cut-off value was identified to be 107 cps. The authors conclude that women with ROS levels higher than 107 cps may encounter difficulties conceiving a viable embryo [28]. The result of this study stresses the importance of antioxidants to suppress ROS buildup and maintain physiological levels of free radicals for proper cell functioning and homeostasis.

2.1. Unfavorable Balance of ROS versus Antioxidants in Endometriosis. Researchers have examined that high levels of ROS can impact fertility in patients with endometriosis. Szczepańska et al. compared the antioxidant levels of superoxide dismutase in the peritoneal fluid in infertile women with (1) endometriosis, (2) unexplained infertility, and (3) fertile women. The lowest concentrations of superoxide dismutase were found in the first group of patients [29]. Similar findings were reported by Liu et al. who demonstrated decreased amounts of superoxide dismutase in the peritoneal fluid of infertile women with endometriosis when compared with fertile women [30]. Therefore, an imbalance between ROS levels and antioxidants can explain infertility in patients with endometriosis. In a study by Prieto et al., the concentration of vitamin C was found to decrease in the follicular fluid of patients with endometriosis compared to infertile patients without endometriosis. This could be due to the excessive consumption of the antioxidant in order to neutralize the abnormal levels of ROS [31]. Furthermore, decreased levels of vitamin C have been associated with ovarian atrophy and follicular atresia implicating its importance in female infertility [32]. Although ROS levels were determined to be crucial in promoting infertility in patients with endometriosis, there is little agreement on oxidative stress markers, due to the inconsistency in results across different studies [31]. According to Jackson et al., higher concentration of Vitamin E was reported in the follicular fluid of patients with endometriosis [33]. Conversely, concentration of vitamin E was lower in the peritoneal fluid of patients with endometriosis [34]. This discordance in results may be explained by the vitamin E rich diet or the nutrient supplementation that is commonly suggested for women suffering from endometriosis [31]. It may also be due to the increased consumption of vitamin E in the peritoneal fluid window.

Retrograde menstruation is another theory for endometriosis and seems to also lead to increased oxidative stress through a different mechanism. It may also be observed in women with normal menstruation and can subsequently

cause an increase in iron levels in the female pelvis. The increased iron is taken up by macrophages, therefore reducing the ability of ferritin to remove this heavy metal. The lack of antioxidant activity of ferritin therefore leads to oxidative stress. The increase in oxidative stress causes change in the cellular function due to changes in the genetic and protein content and consequently, the release of inflammatory molecules [10]. Endometriosis has been reported to be associated with worsening of IVF outcomes, due to impaired oocyte quality, decreased fertility, and compromised implantation rates. The increase in ROS in endometriosis patients can lead to adverse effects on embryo such as intrauterine growth restriction, abortions, or fetal dysmorphogenesis [35]. DNA damage was the highest in granulosa cells from patients with endometriosis which resulted in fewer good quality embryos in patients undergoing IVF [36].

2.2. Increased ROS Levels in Polycystic Ovarian Syndrome. Oxidative stress has been implicated in different female diseases including polycystic ovarian syndrome (PCOS). Various studies reflect the presence of oxidative stress in PCOS patients. In a study by Hilali et al., PCOS patients had increased serum prolidase activity as well as total oxidant status and oxidative stress index, which is the ratio of oxidants to total antioxidants status. Prolidase is a matrix metalloproteinase (MMP) that degrades the extracellular matrix. It was found that increased MMPs can result in symptoms seen in women with PCOS such as abnormal ovarian extracellular remodeling, multiple cyst formation, and chronic anovulation, leading to infertility [37].

Furthermore, infertility in PCOS patients was related to the effect of follicular fluid's oxidative stress levels on the meiotic spindle formation in the oocyte. Fertilization rates and embryo quality were found to be decreased in these women, when compared to women suffering from tubal factor disease and accompanied by increased levels of ROS. These data and other studies indicate that elevated ROS in the follicular fluid tends to limit the fertilization potential of oocytes, through disruption of meiotic spindle formation [38].

2.3. Association of Increased ROS Levels in Unexplained Infertility. By definition, unexplained infertility is considered as a "diagnosis of exclusion" when no known factors are responsible for infertility [39]. Studies utilizing malondialdehyde (MDA), a marker of lipid peroxidation [40], have implicated the role of oxidative stress in patients with unexplained infertility. Conversely, it was also seen that glutathione was decreased in the peritoneal fluid of patients with idiopathic infertility [41]. Hence, one theory is that underlying cause of unexplained infertility is the disruption of the delicate balance that exists between ROS and antioxidant levels [41].

Selenium is part of the selenoenzyme glutathione peroxidase and protects the integrity of membrane structures by enzymatically degrading endogenous and exogenous hydrogen peroxide and lipid peroxides. Selenium levels in follicular fluid of infertile patients who presented for IVF treatment were measured [42]. The women were divided into three groups depending on the etiology of infertility: tubal factor,

unexplained, and male factor. Selenium and glutathione peroxidase levels were found to be the lowest in patients with unexplained infertility. Consequently reduced amount of antioxidants in ovarian follicle could be a reason for unexplained infertility [42].

3. Power of Proteomics in Studies of Female Infertility

Post-translational modifications occurring within cells are mainly responsible for the discrepancies noted between the genome and the expressed proteome. Changes that occur at the gene level are not always representative of the processes taking place inside the cell, as the proteins exert their multitudes of functions via more complex and diverse structures, generated through posttranslational modifications and protein-protein interactions [13]. Currently, ~300 different types of PTM are responsible for the huge repertoire of proteins originating from a small number of genes [43]. This leads to diversity in the function and number of proteins, far more complex than visualized by the number of genes identified by the human genome project. It is, however, the proteins that finally dictate the function of each cell through dynamic interactions with their environment and with each other [15]. Attention has now shifted to the protein complement of the genome, as the one gene-one protein concept that had served as a landmark in molecular biology is no longer true.

With the advancement in technology for studying proteins, it has become possible to focus on the role of proteomics in different fields of medicine. The word “omics” refers to the study of a certain field, in this case the proteins under investigation. This includes study of the protein content of a cell or a tissue, providing insight and ideas for biomedical interventions and identification of potential biomarkers [14]. Proteins differ greatly between two cells, between the same cell at different times and between normal and abnormal cells. Complexity in protein function has driven investigators to probe the protein factors responsible for both male and female infertilities by studying tissues and bodily fluids of the reproductive tracts as well as in pathological tissues. Also, proteomic analysis could aid in the identification of potential “fertility proteins” that could prove highly beneficial in the diagnosis of infertility. In this era of proteomics, it is important that proteomic experiments are reproducible from one lab to the other. Standard procedures for sample collection, handling, storage, and analysis need to be adopted, as they will be important for identification of biomarkers in disease development and progression [44].

Advanced research has led to a better understanding of the underlying causes and the mechanistic pathways of female infertility [45]. Numerous studies have been conducted on endometrial tissue and secretions, endometrial receptivity, ovaries, uterine fluid, follicular fluid, peritoneal fluid, plasma, endometriotic tissue, and tissues from PCOS patients [8, 13, 31, 46–48]. However, the collection of reproductive samples is a challenge as some of the samples have to be obtained invasively [44]. The bigger challenge is the

analysis of the diverse microenvironments since they not only contain proteins of interest, but also growth factors, inflammatory factors, different cells, and several biological molecules. This is a limitation in proteomics as the samples have very high concentration of plasma proteins such as albumin that should be removed in order to determine the protein content of plasma related to endometriosis [47]. Another limitation is the retrieval of adequate amount of the sample and hence the need for techniques with high specificity and sensitivity to measure small amounts of proteins [48].

The ability of proteomics to characterize certain body fluids and determine their contents makes it a powerful tool in today's world [48]. It can be used for the determination of biomarkers specific to certain diseases as well as to differentiate between fertile and infertile women. The search for certain proteins that can discriminate a receptive endometrium from one that is less receptive continues, as it has great potential in increasing the success rates associated with natural fertility as well as in assisted reproductive techniques. Considerable progress has been made in classifying embryonic proteins, which will aid single embryo selection during assisted reproductive techniques, thereby making the process of ART more efficient and decreasing unwanted multiple gestation rates as well as failed procedures [4].

4. Methodology of Proteomic Analyses in Infertile Females

The separation and identification of proteins are currently performed using different methods depending on the requirements of each experiment. Techniques are continuously modified to achieve more accurate and specific results. Physical factors such as molecular mass, acidity, charge, and amino acid content of proteins determine the design of the experiment.

The most commonly used procedures are a systematic two-dimensional gel electrophoresis (2-DE) for separating the proteins, mass spectrometry for identifying them, and Western blotting for confirmation. Integral to the purity of samples, it is essential that before separation, samples should be denatured, purified, and solubilized for complete disintegration of internal bonds resulting in single proteins [49].

4.1. Two-Dimensional Gel Electrophoresis (2DE) and Difference Gel Electrophoresis (DIGE). 2DE is an integral tool in the study of proteomics. It is a classical method often described as the proteomic “workhorse.” It enables the separation of complex protein mixtures by combining isoelectric focusing (IEF) in the first dimension and SDS PAGE in the second dimension [50]. Protein profiles can be visualized using both fluorescent and visible stains. It is usually used to find biomarkers in various disease states. 2DE has poor reproducibility and limited sensitivity.

Difference gel electrophoresis (DIGE), in which two protein samples are separately labeled with different fluorescent dyes and then coelectrophoresed on the same 2DE gel, was developed to overcome the reproducibility and

sensitivity limitations. DIGE utilizes mass- and charge-matched, spectrally resolvable fluorescent dyes (Cy2, Cy3, and Cy5) to label up to 3 different protein samples (or 2 samples and 1 pooled internal standard) *in vitro* prior to 2D electrophoresis [51]. The fluorescent dyes have been designed so that they do not alter the charge of proteins or impart significant electrophoretic mobility differences to identical proteins. Both the control and the experimental samples are run in a single polyacrylamide gel but imaged separately (Typhoon 9410) at a discrete wavelength for each respective dye. Hence, the images are overlaid without any “warping” which substantially raises the confidence with which protein changes between samples can be detected and quantified.

Quantitative and comparative proteomics can be done using 2D-DIGE (two-dimensional difference gel electrophoresis). An automated software program is used to detect, quantify, and annotate differentially expressed proteins. 2D-DIGE offers all the advantages of 2D-PAGE and overcomes the inherent disadvantage of variation and reproducibility problem in a 2D-PAGE. These procedures are powerful technologies for comparing complex protein mixtures from biological samples in proteomics research. Compared to traditional 2-D gel techniques for protein profiling, DIGE provides a significantly more efficient, sensitive, and reliable way to detect proteins whose expression is altered between control and treated samples.

There are many advantages to 2D-DIGE over traditional 2D gels: (1) high sensitivity: CyDye fluorescent dye has a sensitivity of 0.2 ng/spot versus 100 ng/spot with the traditional 2D gels stained by Coomassie; (2) high accuracy: extremely high spot resolution allows accurate software-aided spot quantitation and protein expression comparison between samples. Differences as small as 10% can be detected and protein isoforms and posttranslational modifications can be easily visualized by 2D-DIGE. While DIGE provides a reliable and sensitive platform for discovering proteome changes in an unlimited variety of circumstances, conventional 2DE is still needed to identify the proteins [52, 53].

4.2. Mass Spectrometry. The principle of mass spectrometry is applicable to all molecules including lipids and proteins. In proteomics principle of mass spectrometry depends on the separation of the peptides according to their mass-to-charge ratio. An ionizer bombards the proteins with hydrogen ions and results in a charge on the protein as they pass through the mass analyzer to the detector which is attached to a computer program [54].

For conducting gel-based proteomics studies, the region of gel containing the protein of interest is excised, and the sample is digested “in-gel” with trypsin (which cleaves proteins on the C-terminal side of either lysine or arginine), the proteins are then eluted from acrylamide. These peptides are then subjected to MALDI-TOF, producing a series of peaks, each describing the molecular mass of a single peptide in the mixture.

Proteomic application of mass spectrometry (MS) is constantly growing as more improved instruments and smarter ways to couple them become available. The most commonly used mass analyzers for protein biochemistry applications are

time-of-flight (TOF), triple-quadrupole, quadrupole-TOF, ion trap instruments, and hybrid ion-trap orbitrap instrument.

Four common uses of MS based proteomics are (1) protein identification, (2) protein sequencing, (3) identification of posttranslational modifications, and (4) characterization of multiprotein complexes. There are other complex MS analyzers such as the Fourier transform-ion cyclotron resonance MS (FT-ICR). There are also other MS applications, such as structural analysis. The principle of mass spectrometry is applicable to all molecules including lipids and proteins.

4.3. Matrix Assisted Laser Ionization/Desorption Mass Spectrometry. In the tissues from patients with endometriosis, protein bands are excised and subjected to further identification using one of the most common ionization techniques used in biology which are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). MALDI is most commonly used to produce a preliminary scan of the peptide components released from a sample by proteolytic digestion, such as the proteins present in an acrylamide gel slice. It is a solid phase technique in that researchers mix a peptide sample with a huge excess of matrix material, usually either α -cyano-4-hydroxycinnamic acid or dihydrobenzoic acid, and precipitate the mixture on a plate by drying. Whereas MALDI is a solid-state and pulsed process, ESI is a liquid phase and is usually continuous technology. ESI is compatible with both high-pressure liquid chromatography (HPLC) and capillary electrophoresis, both of which may be used to concentrate and purify individual peptides prior to mass analysis. MS instruments, despite their names, do not actually provide a mass value. Instead, they report the mass-to-charge (m/z) ratio. If the charge of the ion is known, the mass can be calculated. The most commonly used mass analyzers for protein biochemistry applications are time-of-flight (TOF), triple-quadrupole, quadrupole-TOF, ion trap instruments, and hybrid ion trap Orbitrap instruments. The TOF analyzer is conceptually the simplest spectrometer [54]. Typical configurations for biological applications are MALDI-TOF and ESI coupled to an ion trap, triple-quad, Q-TOF, or orbitrap.

4.4. Surface-Enhanced Laser Desorption/Ionization. Surface-enhanced laser desorption/ionization (SELDI) is an ionization method in mass spectrometry that is used for the analysis of protein mixtures. SELDI is typically used with time-of-flight mass spectrometers and is used to detect proteins in tissue samples, blood, urine, or other clinical samples. Comparison of protein levels between patients with and without a disease can be used for identification of new biomarkers. SELDI-TOF MS is a novel approach to biomarker discovery that combines two powerful technologies: chromatography and mass spectrometry. SELDI-TOF-MS is a variation of matrix-assisted laser desorption/ionization (MALDI) that uses a target modified to achieve biochemical affinity with the analyte compound.

In SELDI, the protein mixture is spotted on a surface modified with a chemical functionality. Some proteins in the

sample bind to the surface, while the others are removed by washing. After washing the spotted sample, the matrix is applied to the surface and allowed to crystallize with the sample peptides. Binding to the SELDI surface acts as a separation step and the subset of proteins that bind to the surface are easier to analyze. SELDI technology was commercialized in 1997 as the Protein Chip system and is marketed by Bio-Rad Laboratories. The Protein Chip platform consists of chips presenting specific chromatographic surfaces, including reverse phase, anionic exchange, cationic exchange, and immobilized metal affinity surface.

SELDI has been applied in identifying diagnostic markers in ovarian, prostate, breast, bladder, hepatic, and pancreatic cancer using serum or plasma. The technique has also been used to characterize phosphorylated and glycosylated proteins, transcription factors, and peptides and proteins shed or secreted by various cancer cell lines. SELDI-TOF-MS is a technology that can produce proteomic “fingerprints” from biological samples using a relatively high throughput platform. Posttranslationally modified proteins can also be detected using SELDI-TOF MS.

An application that has generated much interest in SELDI-TOF MS is its promise as a diagnostic tool in the early detection of diseases, such as ovarian cancer. Serum samples taken from healthy and diseased subjects are spotted onto protein chips and analyzed by TOF MS to generate a protein profile.

SELDI-TOF MS has its limitations. The system is manual, time consuming, and prone to human error. The low resolution, and hence mass accuracy, coupled with the inability to do TOF MS/MS, prevent reliable identification based on conventional bioinformatic searching. Although a SELDI interface is available for higher-resolution instruments, such as the hybrid quadrupole TOF instrument that has a much higher mass accuracy and MS/MS capabilities, the routine application of these instrument platforms for biomarker identification directly from the applied sample has yet to be demonstrated. Largely successful at discovering proteins in the low-molecular-weight range, SELDI-TOF MS has not yet shown itself consistently successful in studying high-molecular-weight proteins.

SELDI-TOF MS provides a simple, low-resolution pattern generated from proteins retained on a specific chromatographic surface. In most instances, it does not allow the direct identification of proteins that may be potential disease biomarkers. SELDI-TOF MS can rapidly screen and generate proteomic patterns for hundreds of crude samples. This simple answer may have a profound impact on how proteomics is viewed in the future.

Bias in serum specimens of early studies, differences in study design, and limitations of proteins detected by SELDI-TOF MS in unfractionated serum may explain the inability of this study to identify patients especially in studies on patients with colorectal cancer.

There are many methods of mass spectrometry-based proteomics that do not use TOF. For example, the Q-Exactive (Thermo) utilizes a quadrupole-orbitrap hybrid mass spectrometer and is the most widely used platform for shotgun proteomics. Waters' MSE platform is based on a very

good TOF analyzer but depends on a Quadrupole analyzer (hybrid) in line. Moreover, the introduction of ion mobility is one of the backbones of their technology. The newer Triple TOF (AB Sciex) is in essence a quadrupole-time of flight hybrid as well. Even FT-ICR MS has been used due to its very high resolution.

4.5. Liquid Chromatography-Mass Spectrometry. Liquid chromatography-mass spectrometry (LC-MS) is a hyphenated technique, which combines the separation power of LC with the detection power of mass spectrometry. Liquid chromatography-mass spectrometry (LC-MS, or alternatively HPLC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS). The LC-MS system consists of four main components: (1) a chromatographic column, (2) an ionization source, (3) mass analyzer, and (4) detector. LC-MS is a powerful technique that has very high sensitivity and selectivity and so is useful in many applications.

Liquid chromatography/mass spectrometry (LC/MS) has become a powerful technology in proteomics studies in drug discovery, including target protein characterization and discovery of biomarkers. Its application is oriented towards the separation, general detection, and potential identification of chemicals of particular masses in the presence of other chemicals (i.e., in complex mixtures), for example, natural products from natural-products extracts and pure substances from mixtures of chemical intermediates.

LC-MS is also used in proteomics where again components of a complex mixture must be detected and identified in some manner. The bottom-up proteomics LC-MS approach generally involves in-solution protease digestion and denaturation. The denaturation of the tertiary structure is done using urea, trypsin enzyme is used for the degradation and the remaining cysteine residues are capped with iodoacetamide. They are then subjected to LC-MS/MS (tandem MS) to derive their individual sequences. LC-MS/MS is the most commonly used for proteomic analysis of complex samples where peptide masses may overlap even with a high-resolution mass spectrometer. There are two general approaches to LC-MS/MS analysis of complex peptide mixtures. The first is data-dependent experiments that involve MS scans followed by MS/MS analysis on the most abundant peptide ions [55]. These experiments are utilized widely in proteomics and involve the MS/MS analysis of only one peptide ion at a time.

For complex mixtures, a data-dependent analysis may not be able to sequence all of the peptides present in the sample which has led to the development of data-independent acquisition, LC-MSE. These experiments involve the MS/MS analysis of wide mass ranges resulting in the fragmentation of multiple precursors in a single scan. These experiments have been shown to provide more comprehensive protein identification [56]. Samples of complex biological fluids like human serum may be run in a modern LC-MS/MS system and result in over 1000 proteins being identified, provided that the sample was first separated on an SDS-PAGE gel or

multidimensional HPLC (i.e., SCX fractionation prior to LC-MS/MS analysis).

Within a little more than a decade LC-MS/MS has transformed from an esoteric gold standard technology to an affordable, flexible, and accessible technique for most clinical laboratories. LC-MS/MS has helped in developing routine methods of high sensitivity, high specificity, high throughput, and high cost effectiveness in biochemical genetics/newborn screening, drug and toxicology testing, and endocrine testing of steroids and biogenic amines. These novel methods have made a positive impact on patient care on both economic and quality fronts. LC-MS/MS has been applied to known, established peptides and proteins biomarkers that is, targeted proteomics.

Several key limitations of LC-MS/MS have become apparent with the exponential increase of its use in clinical laboratories. They center on the following, interacting aspects of clinical LC-MS/MS: highly manual workflows, complexity of operation and maintenance of instrumentation, sample throughput limits, insufficient detection sensitivity for some analytes, and problems with detection specificity.

LC/MS plays important roles in protein structural identification and quantitative measurements in proteomics research, an integral part of the drug discovery process. Its broad applications include target protein characterization and biomarker discovery in addressing challenging issues related to drug efficacy and safety in drug discovery environment. New technological advances in this area can provide additional capabilities in using LC/MS for proteomics studies. The ability to identify proteins increases 10-fold (when comparing gel-free to gel) at least, and quantification is much more precise.

Another emerging field in proteomics research is top-down proteomics. In contrast to bottom-up experiments, intact proteins are analyzed directly using high-resolution MS and dissociated subsequently by a tandem mass spectrometer. Dissociation techniques used in top-down proteomics include collision induced dissociation (CID), electron-capture dissociation (ECD), and electron-transfer dissociation (ETD). The use of LC/MS in a top-down approach has been reported in the literature, demonstrating feasibility of this method in obtaining data on a chromatographic time scale for the first time.

Orbitrap-based mass spectrometers became a workhorse in proteomics and are also widely used in all major applications of life science mass spectrometry such as metabolism, metabolomics, environmental, food, and safety analysis. Orbitrap LC-MS technology is the recognized standard for accurate mass and high-resolution measurement. Orbitrap LC-MS technology routinely delivers the highest-resolution and mass accuracy necessary to reduce analysis times and increase confidence in results. Combined with superior dynamic range and unsurpassed sensitivity, Orbitrap platforms are the only technology available that is capable of providing all four benefits at the same time, without compromise.

4.6. Protein Identification and Database Searching. Once the molecular masses of the unknown proteins are established experimentally, they are compared to predetermined

molecular masses of previously sequenced proteins from the reference databases. Peptide mass fingerprinting (PMF) (also known as protein fingerprinting) is an analytical technique for protein identification. The unknown protein of interest is first cleaved into smaller peptides, whose absolute masses can be accurately measured with a mass spectrometer such as MALDI-TOF or ESI-TOF. These masses are then compared to either a database containing known protein sequences or even the genome. The peptide masses are compared to protein databases such as Swissprot, which contain protein sequence information. Software performs *in silico* digests on proteins in the database with the same enzyme (e.g., trypsin) used in the chemical cleavage reaction. The mass of these peptide fragments is then calculated and compared to the peak list of measured peptide masses. The results are statistically analyzed and possible matches are returned in a results table.

Alternative methods involve the LC-MS/MS analysis of digested proteins. These experiments are carried out in a data-dependent manner which involves initial MS analysis to determine peptide molecular weight which is followed by MS/MS analysis of each peptide to determine the peptide sequence. The LC-MS/MS data is searched against a protein database which has been subjected to a theoretical digestion and the theoretical MS/MS spectra are generated for each peptide. Search programs such as Mascot, Sequest, X! Tandem, and Andromeda can then compare the experimentally observed MS/MS spectrum to the predicted peptide spectra and determine which peptides, and therefore which proteins, are the best matches. Coupling LC-MS/MS with database searches can result in 1000's of protein identifications from a single experiment [57].

There are a variety of databases available: UniProt is a comprehensive, high-quality, and freely accessible database of protein sequence and functional information, with many entries being derived from genome sequencing projects. It contains a large amount of information about the biological function of proteins derived from the research literature. The UniProt consortium comprises the European Bioinformatics Institute (EBI), the Swiss Institute of Bioinformatics (SIB), and the Protein Information Resource (PIR). UniProt provides four core databases:

- (1) UniProtKB (with subparts Swiss-Prot and TrEMBL), UniParc, UniRef, and UniMes;
- (2) UniProt Knowledgebase (UniProtKB) is a protein database partially curated by experts, consisting of two sections: UniProtKB/Swiss-Prot (containing reviewed, manually annotated entries) and UniProtKB/TrEMBL (containing unreviewed, automatically annotated entries);
- (3) UniProtKB/Swiss-Prot is a manually annotated, non-redundant protein sequence database. It combines information extracted from scientific literature and biocurator-evaluated computational analysis. The aim of UniProtKB/Swiss-Prot is to provide all known relevant information about a particular protein;

- (4) UniProtKB/TrEMBL contains high-quality computationally analyzed records, which are enriched with automatic annotation.

UniProt Archive (UniParc) is a comprehensive and non-redundant database, which contains all the protein sequences from the main, publicly available protein sequence databases.

In addition there are other databases such as the UniProt Reference Clusters (UniRef) that consist of three databases of clustered sets of protein sequences from UniProtKB and selected UniParc records. The UniProt Metagenomic and Environmental Sequences (UniMES) database is a repository specifically developed for metagenomic and environmental data.

UniParc contains protein sequences from the following publicly available databases: UniProtKB/Swiss-Prot, UniProtKB/Swiss-Prot protein isoforms, UniProtKB/TrEMBL; Vertebrate and Genome Annotation Database (VEGA), and WormBase.

Publicly available (Gene Ontology (GO) annotations from GO Term Finder and GO Term Mapper), UNIPROT, STRAP, and proprietary software packages including Ingenuity Pathway Analysis (IPA, Ingenuity Systems) and Metacore (GeneGo Inc.) as well as the STRING database and Cytoscape are available to identify the differentially affected processes, pathways, cellular distribution, and protein-protein interactions amongst proteins in the control and experimental groups, as well as aid in data integration [58–62].

4.7. Validation of Identified Proteins. Western blot or the protein immunoblot is a widely used analytical technique used to detect specific proteins in a sample of tissue homogenate or extract. Other related techniques include dot blot analysis, Zestern analysis, and immunohistochemistry where antibodies are used to detect proteins in tissues and cells by immunostaining and enzyme-linked immunosorbent assay (ELISA).

Biomarker validation experiments can also be performed using multiple reactions monitoring (MRM) peptide quantitation on an LC-MS/MS platform [60]. These experiments differ from those described above in that peptides from the proteins of interest are analyzed by MS/MS. Typically, three peptides are selected for each protein; up to 50 proteins can be analyzed in a single analysis, which allows proteins quantitation to be performed on multiple samples in a reasonable time frame. In addition, MRM analysis coupled with isotope dilution mass spectrometry allows for the absolute quantitation of protein abundances.

5. Proteomic Profiles of Females with Endometriosis

The presence of endometrial glands and stroma outside the uterine cavity is referred to as endometriosis [63]. This disease is benign and its development is primarily estrogen-dependent [47]. The manifested symptoms of endometriosis are most commonly pelvic pain and infertility as the lesions develop and establish within the peritoneal region. Although there is a delay in the diagnosis of endometriosis, it has been

noted that the most common symptoms are dysmenorrhea, pelvic pain, dyspareunia, and infertility [48]. A diagnosis of endometriosis is established in 17% of women who present with primary infertility, 5–21% of women with pelvic pain, and 50% of adolescents with dysmenorrhea [64]. These statistics are evidence of the prevalence of this disease among women of a wide age range, making endometriosis one of the most investigated gynecological disorders.

The prevalence of endometriosis is the highest among women of reproductive age group and declines with age, making it a topic of interest to researchers [65]. It was noted that an early diagnosis of the disease greatly reduces the risks and symptoms associated with the disease. However, this has proven to be more challenging than initially thought since the only method of diagnosis of endometriosis is by invasive techniques such as laparoscopy [47]. General fear of medical intervention prevents women from undergoing the procedure unless they are symptomatic or have already been diagnosed. According to Husby et al., the time of delay between pelvic pain and final diagnosis of endometriosis was a mean of 6.7 years [66]. However, since studies have shown that endometriosis is progressive in 50% of the cases, early diagnosis is the key [47].

Endometriosis can be classified into three types, each of which affects a different region or tissue of the abdomen. Ovarian endometriosis and peritoneal endometriosis appear as multiple lesions of the endometrial tissue on the ovaries or peritoneum, respectively. Endometriosis of the rectovaginal septum is a more invasive form of endometriosis which involves the infiltration of adenomyotic tissue, making this a more persistent form of the disease [8]. Each of the subtypes has its unique identity due to differences in presentation and underlying pathology. The link between endometriosis and infertility is not completely clear yet. Different reasons have been proposed that could be responsible for infertility such as changes in the peritoneal fluid content and alterations of granulosa cells as well as changes in the follicular fluid including immunological changes.

The high prevalence of ROS in endometriosis patients, due to the oxidant-antioxidant imbalance mentioned previously, results in DNA damage of the sperm that is incubated with peritoneal fluid from endometriosis patients. Although regulated by different factors, the oocyte DNA exhibits similar changes when subjected to incubation with peritoneal fluid from endometriosis patients. Alterations in oocyte DNA and oocyte competence were seen when co-incubated with peritoneal fluid from endometriosis patients. Change in endometrial receptivity also plays a role in the resulting infertility in women with endometriosis [67].

The search for molecular markers to identify the symptoms of endometriosis early on in disease development continues, as it will not only aid in preventing progression of the disease but also improve the quality of life, fertility status, and health of women suffering from endometriosis worldwide [68]. Numerous proteins have been identified in different studies (Table 1) and this review will only focus on a few that are either linked to or altered by oxidative stress. According to Fassbender et al., different proteins are expressed at different stages of the menstrual cycle depending on the severity of endometriosis [47].

TABLE 1: Proteins identified in relation to endometriosis.

Protein assessed/technique of assessment	Function and site of expression	Source	Association with endometriosis	Population size	Reference
Fibrinogen B peptide/SELDI-TOF-MS	Helps in blood coagulation, regulation of cell adhesion, and spreading.	Plasma	Decreased in endometriosis due to overconsumption and the consequence is adhesion of endometrial fragments.	254 samples from women who underwent laparoscopy with or without pelvic pain. 89 women without endometriosis and 165 with endometriosis	[47]
Afamin/ELISA or 2D-gel electrophoresis	Vitamin E binding protein.	Peritoneal fluid	Increased causing a decrease in antioxidant activity	242 reproductive age women. [65] 24 samples of peritoneal fluid from symptomatic women	[8, 69]
Hemopexin/2Dgel electrophoresis	Heme binding protein	Peritoneal fluid	Decreased leading to oxidative stress	24 samples of peritoneal fluid from symptomatic women	[8]
Inter-alpha inhibitor H4 (ITI4)/2Dgel electrophoresis	Not clear but involved in acute phase inflammation	Peritoneal fluid	Increased in peritoneal endometriosis.	24 samples of peritoneal fluid from symptomatic women	[8]
Apolipoprotein A4/2Dgel electrophoresis	Bind lipids to form lipoproteins.	Peritoneal fluid	Decreased in ovarian endometriosis	24 samples of peritoneal fluid from symptomatic women	[8]
Haptoglobin/2Dgel electrophoresis	Hemoglobin binding protein	Peritoneal fluid	Increased. Removes iron and prevents oxidative stress	24 samples of peritoneal fluid from symptomatic women	[8]
Vitronectin/2Dgel electrophoresis	Involved in cell migration, adhesion, and invasion.	Peritoneal fluid	Increased	24 samples of peritoneal fluid from symptomatic women	[8]
SERPINA/2Dgel electrophoresis	Associated with inflammatory outcomes	Peritoneal fluid	Increased and might be a good biomarker	24 samples of peritoneal fluid from symptomatic women	[8]
Superoxide dismutase (SOD)/mass spectrometry, high performance liquid chromatography, and ELISA	Breakdown of superoxide radical into hydrogen peroxide and oxygen	Follicular fluid	Decreased leading to oxidative stress	91 infertile women who were going to undergo IVE, 23 with endometriosis identified by laparoscopy or ultrasound and 68 infertile due to different reasons (not endometriosis)	[31]
Thioredoxin binding protein 2 (TBP)/ELISA	Binds thioredoxin (TRX) which is an antioxidant	Cells from endometrial tissue	It was noted that there is an increase in the TRX/TBP in endometriosis patients	66 women 35 with confirmed endometriosis and 31 patients without endometriosis as a control	[70]
Interleukins (IL-6, IL-10, and IL-1B)	Function in the immune system of different cells.	Serum and follicular fluid	Increase in follicular fluid showing immune involvement with the disease	38 patients, 20 with endometriosis, and 18 controls	[71]
Vascular endothelial growth factor/ELISA	Increase in vascular permeability	Peritoneal fluid	Increases in patients with endometriosis	43 patients in reproductive age who were undergoing diagnostic laparoscopy for dysmenorrhea or elective laparoscopy for infertility, 19 controls without endometriosis, and 24 patients with endometriosis	[72]
G antigen family B/Mass spectrometry and 2Dgel electrophoresis	Responsible for the androgen-insensitive phenotype	Serum	May effect the level of estrogen and therefore the occurrence of endometriosis	12 women of reproductive age, 6 with laparoscopically proven endometriosis, and 6 without endometriosis	[73]

TABLE 1: Continued.

Protein assessed/technique of assessment	Function and site of expression	Source	Association with endometriosis	Population size	Reference
Carbonic anhydrase 1/mass spectrometry and 2D-gel electrophoresis	Involved with the process of bio-respiration	Serum	Decreased in patients with endometriosis	12 women of reproductive age, 6 with laparoscopically proven endometriosis, and 6 without endometriosis	[73]
Actin family proteins (actin related protein 6 and actin-like-7-alpha)/mass spectrometry and 2D-gel electrophoresis	Actin is in the cellular cytoskeleton and involved in mitosis, signal transduction, and motility.	Serum	Differentially expressed in endometriosis	12 women of reproductive age, 6 with laparoscopically proven endometriosis, and 6 without endometriosis	[73]
CD166 antigen/mass spectrometry and 2D-gel electrophoresis	Cell adhesion molecules that bind to CD6 and might play a role in T and B cell adhesion to leukocytes	Serum	Downregulated causing change in immune function of endometriosis patients	12 women of reproductive age, 6 with laparoscopically proven endometriosis, and 6 without endometriosis	[73]
CyclinA1/mass spectrometry and 2D-gel electrophoresis	Controls the cell cycle at G1/S and the G2/M stops	Serum	Differentially expressed	12 women of reproductive age, 6 with laparoscopically proven endometriosis and 6 without endometriosis	[73]
Vimentin/mass spectrometry and 2D-gel electrophoresis	A component of the cytoskeleton especially in mesenchymal cells	Serum	Increased in endometriosis tissue as cytoskeletal changes are required	12 women of reproductive age, 6 with laparoscopically proven endometriosis, and 6 without endometriosis	[73]
B-actin/mass spectrometry and 2D-gel electrophoresis	Component of the cytoskeleton and mediates cell motility and may affect expression of some endometriosis related genes	Serum	Increased as cellular changes occur	12 women of reproductive age, 6 with laparoscopically proven endometriosis, and 6 without endometriosis	[73]
ATP synthase/mass spectrometry and 2D gel electrophoresis	The production of ATP from ADP	Serum	Increased as retrograde menstruation requires ATP	12 women of reproductive age, 6 with laparoscopically proven endometriosis and 6 without endometriosis	[73]
Glycodelin/2D-gel electrophoresis	Involved in regulating uterine environment for pregnancy and timing the occurrence of fertilization events	Endometrial tissue	Increased in the eutopic endometrium of patients	24 women undergoing laparoscopy either for endometriosis, elective tubal sterilization, or diagnostic purposes.	[12]
HSP90/2D-gel electrophoresis	Chaperone molecule for the correct folding of proteins	Endometrial tissue	Decreased expression in eutopic endometrium	24 women undergoing laparoscopy either for endometriosis, elective tubal sterilization, or diagnostic purposes	[12]
Annexin A2/2D-gel electrophoresis	Involved in the cytoskeleton and exocytosis	Endometrial tissue	Decreased in patients showing less secretory activity	24 women undergoing laparoscopy either for endometriosis, elective tubal sterilization, or diagnostic purposes.	[12]
Peroxiredoxin/2D-gel electrophoresis	Antioxidant molecules	Endometrial tissue	Altered expression in endometrial tissue	24 women undergoing laparoscopy either for endometriosis, elective tubal sterilization, or diagnostic purposes.	[12]

Seeber et al. were able to identify and validate differentially expressed proteins in the plasma of endometriosis patients. The use of highly accurate methods such as SELDI-TOF-MS may help to potentially avoid laparoscopic procedures, unless absolutely required [48]. In one study, the levels of fibrinogen B peptide were compared in women who underwent laparoscopic procedures with or without pelvic pain. The samples studied were from women in the luteal phase of the menstrual cycle. Fibrinogen B peptide (Table 1) was decreased in endometriosis patients when compared to the control group suggesting its potential use as a biomarker [47]. The conversion of fibrinogen into fibrin is one of the several steps in the blood clotting cascade, after vascular injury. Fibrinogen is also involved in cellular processes, inflammation, and wound healing. It was postulated that the decrease of fibrinogen B chain in the plasma of endometriosis patients indicated that more fibrinogen was converted into fibrin in the peritoneal fluid that led to adhesion and attachment of peritoneal tissue—a hallmark of peritoneal endometriosis [47].

Afamin is a vitamin E-binding protein commonly present in peritoneal fluid. Vitamin E, a nonenzymatic antioxidant, present in nonvascular fluid was found to be decreased in peritoneal fluid of endometriosis patients [74]. Several experiments have shown an increase in levels of Afamin in peritoneal fluid of patients with endometriosis when compared to women who are disease free [8, 74]. Higher level of the binding protein Afamin suggests reduced amounts of available antioxidant to scavenge the free radicals and thereby provides a linkage between endometriosis and oxidative stress.

Iron and its deposits, such as heme and hemosiderin, are typically found in endometriotic lesions [69]. Hemopexin, a heme binding protein, was found to be downregulated in peritoneal fluid of patients proven to have endometriosis [8]. Heme, a breakdown product of erythrocytes, is a source of lipid radicals as it interacts with the lipids present within the plasma membrane, leading to an increase in oxidative stress. Heme is also the source of redox imbalance at the surface of peritoneal epithelial cells [10]. The decrease in hemopexin can therefore lead to oxidative stress as noted in patients of endometriosis. Such radical imbalance also leads to damage in the fallopian tubes and retrograde menstruation, further establishing the role of oxidative stress in the disease [10].

Superoxide dismutase (SOD) is an enzymatic antioxidant produced by the theca interna of ovarian follicles. It catalyzes the breakdown of superoxide radical into hydrogen peroxide and oxygen thereby producing less reactive molecules. Prieto et al. showed that this protein was decreased in the follicular fluid of women with endometriosis ($P = 0.05$), further highlighting the relation between oxidative stress and the disease [31]. SOD is only present in ovaries, particularly in the follicular fluid, providing protection for the theca interna. The follicular fluid is located in a compartment surrounded by highly vascularized cells that act as a blood-follicle barrier [75]. The microvasculature within the theca interna layer is responsible for transferring the SOD from within the cells to the follicular fluid; hence the cells have a protective role.

Thioredoxin (TRX) (Table 1) regulates the levels of oxidative stress in a cell due to its antioxidant activity. It is also

involved in multiple cellular processes including proliferation and apoptosis. Thioredoxin binding protein 2 (TBP-2) regulates the activity of thioredoxin and enhances apoptosis in cells with high oxidative stress. Seo et al. noted that there is an increase in TRX/TBP cellular content in endometriosis patients ($P < 0.001$). The study included 66 women (35 with confirmed endometriosis and 31 patients without endometriosis as a control) in which real-time PCR and immunohistochemistry were used for amplification and localization of the proteins in the endometrial tissue. The levels of TBP and TRX in the serum and peritoneal fluid were determined using ELISA. It was postulated that endometriosis related oxidative stress resulted in a decrease in TBP-2 rather than an increase in TRX and hence the change in the TRX/TBP ratio [70]. The decreased negative regulation of TRX was indicative of lower antioxidant activity and increased oxidative stress. The lower levels of TBP-2 in endometrial cells also indicated lower levels of apoptosis and higher levels of cell proliferation. As a result, uterine cells were implanted in different regions leading to the establishment and progression of the endometriosis [70].

Vascular endothelial growth factor (VEGF) is a polypeptide responsible for blood vessel formation by binding to protein kinase receptors. VEGF has an important role in normal angiogenesis as well as in diseased conditions associated with abnormal vascular proliferation [76]. The levels of VEGF increase in the endometrial tissue of women with endometriosis. Furthermore, levels of VEGF differed at different times of the cycle, being higher during the proliferative phase than the secretory phase in patients with endometriosis as compared with women without endometriosis (control). The level of VEGF in the peritoneal fluid was determined using ELISA. VEGF allows the establishment and proliferation of endometriotic tissue as the polypeptide induces neo-vascularization within and around the tissue [72]. Better understanding of this pathway could potentially assist in treatment options as well as lead to the identification of a biomarker of endometriosis. Antiangiogenic factors have been proposed as therapeutic agents for treatment of women suffering from endometriosis. Antiangiogenic drugs function by inhibiting formation, maintenance, and development of blood vessels and therefore act in hindering the progression of endometriosis [77]. Although studies on the effect of oxidative stress on VEGF in the human endometrium have not been done, the relationship has been studied in various other systems. Studies in gastric cancer by Schäfer et al. have demonstrated that an increase in oxidative stress leads to an increase in VEGF gene expression. Moreover, eliminating reactive oxygen species in turn leads to a decrease in VEGF levels proving the specificity of oxidative stress reactions [78]. The effect of oxidative stress on VEGF has also been studied in embryonic development. ROS was noted to cause changes to transcription factors and hence the pathways leading to increased expression of VEGF and angiogenesis for cell survival [79]. Oxidative stress therefore affects VEGF expression but the link to endometriosis is not clear yet.

The ectopic endometrium found in women with endometriosis is an invasive tissue requiring cytoskeletal rearrangement and modification [73]. Beta-actin (Table 1) is one

such cytoskeletal protein which is a mediator of cell motility and seems to be increased in the serum of women with endometriosis [73]. Such findings lend credibility to the rationale that endometrial cells change location and shape in order to migrate to their new habitats in the diseased state. Other cytoskeletal proteins that have been identified in the tissues of women with endometriosis include alpha-actinin, ezrin, and talin all of which are decreased in the diseased state. All these proteins are involved in cell adhesion and hence in the binding of cells. The downregulation of these proteins is indicative of changes in cellular morphology and the possible movement of cells away from each other [80].

In addition to cytoskeletal changes within the cell, endometriosis is related to the dynamic changes in the extracellular protein expression. The extracellular matrix (ECM) plays an essential role in some of the cell processes such as migration, adhesion, proliferation, and differentiation as well as acts as the basis for the generation of endometriotic tissue [81]. According to Harrington et al., laminin, fibronectin, collagen IV, and vitronectin, all components of the ECM, were expressed with similar patterns in the endometrial tissue of women with no endometriosis but with a regular menstrual cycle and women exhibiting endometriosis. However, Western blotting techniques were only able to detect a change in tenascin which was upregulated in endometriotic tissue as well as the proliferative phase of the menstrual cycle. Although the exact function of this protein is not known, the change in its levels suggests that the higher expression is a result of the factors present in the peritoneal fluid surrounding endometriotic tissue which may aid in the development of endometriosis [81]. In contrast, increase in vitronectin was seen in the peritoneal fluid of patients with peritoneal and ovarian endometriosis [8]. An increase in fibronectin, collagen IV, and laminin was also seen in women with endometriosis. The release of these molecules into the peritoneal cavity during ovulation may result in increased adhesion of endometrial cells that enter the peritoneal cavity due to retrograde menstruation [82]. Furthermore, fibronectin and collagen type IV of the ECM have been shown to enhance cell proliferation [83, 84]. Increase of vitronectin and tenascin in certain cancers led to the postulation that the progression of endometriosis was similar to tumor formation. Studying ECM proteins and their differential expression was proposed to assist into further understanding the etiology of this enigmatic disease [82].

Cyclins are positive regulators of the cell cycle leading to the proliferation of cells. Their activity is associated with that of cyclin-dependent kinases (CDKs) to keep the cell cycle under control. Cyclin A1 was found to be expressed in the testis, promyelocytic cells, and myeloblastic leukemia [85]. In addition, cyclin A1 was found to be differentially expressed in the serum of patients with endometriosis. The association of such a finding with the pathogenesis of the disease is not well established and requires further studies [73]. It has been postulated that in the presence of pathogenic conditions such as endometriosis, there is an increase in oxidative stress which in turn causes damage to the cell cycle control and leads to excessive proliferation of endometrial stromal cells [86].

Heat shock proteins (HSP) accumulate in cells exposed to stressful conditions. They function as cellular chaperones and play an important role in protein folding/unfolding, transportation and assembly of proteins, and cell cycle control and signaling as well as modulation of apoptosis. HSPs are classified according to their molecular weight [87]. HSP 90 modulates the function of some sex steroid receptor proteins. An increase was reported in the levels of HSP 90 during the proliferative phase of the menstrual cycle detected by staining for the protein in the endometrial glands of a normal endometrium retrieved from women who underwent hysterectomy as treatment for cervical intraepithelial neoplasia. This increase mirrored the change occurring at the hormonal level [88]. There are discrepancies reported in the expression levels of HSP90 in endometriosis. While Matsuzaki et al. reported an increase in HSP90 expression, Fowler et al. noted a decrease in the level of the protein in endometrial tissue [12, 89]. The role of HSPs in correcting the damage occurring in the cell allows these small molecules to counteract the effect of oxidative stress and hence potentiates their use as therapeutic agents for various diseases [90]. This theory further correlates with the effect of oxidative stress, as well as the decrease of HSP90 during the progression of endometriosis.

The above proteins are only a sample of the diverse reactions and changes that occur during endometriosis. Antioxidant proteins are underexpressed while those inducing oxidative stress appear to be overexpressed in multiple studies, corroborating the theory that the imbalance of radicals within the body could potentially cause the endometriosis. While levels of Afamin (an antioxidant binding protein) increased in the peritoneal fluid of patients, other authors have shown a decrease in the expression of some proteins that inhibited oxidative stress formation such as hemopexin [10, 74]. Studies from different environments also revealed differential protein expression such as decrease in SOD levels in follicular fluid and an increase in the TRX/TBP ratio in endometrial cells [31, 70]. Proteins with roles secondary to the function of oxidative stress were also altered, including those that cause neovascularization (VEGF) and cytoskeletal proteins that affect cell division, motility, and differentiation [72, 80]. In addition, proteins of the ECM and cell cycle regulators seem to alter the way cells proliferate and may lead to endometriosis [73]. Heat shock proteins, responsible for the circumventing the effects of oxidative stress, are reported to decrease in endometriosis allowing for more cellular damage [12].

6. Role of Proteomics in Polycystic Ovarian Syndrome (PCOS)

PCOS is one of the major causes of infertility and is reported to affect 5–10% of women during their reproductive age [91]. In 1990, National Institute of Health defined polycystic ovarian syndrome (PCOS) as a disease featuring both chronic anovulation and clinical or biochemical signs of hyperandrogenism, that are definitely not due to any other etiology. The presence of polycystic ovaries became one of the required observations for accurate diagnosis of PCOS. It was later noted that PCOS is a symptom of ovarian dysfunction rather

than a diagnosis on its own [92]. The Rotterdam diagnostic criterion for PCOS states that two out of the three listed criteria should be present in order to diagnose a woman with this condition:

- (1) clinical hyperandrogenism (Ferriman-Gallwey score >8) or biochemical hyperandrogenism (elevated total/free testosterone);
- (2) oligomenorrhea (<6 menstrual cycles per year) or oligo-ovulation;
- (3) polycystic ovaries on ultrasound (≥ 12 antral follicles in one ovary or ovarian volume $\geq 10 \text{ cm}^3$) [92].

Hyperandrogenism is commonly seen in PCOS patients due to the elevated LH levels [93]. The condition most commonly manifests itself in the form of hirsutism and acne which may eventually lead to social and psychological distress [91]. 50% of PCOS patients are obese, mostly presenting with an abdominal fat phenotype. This metabolic syndrome eventually leads to the manifestation of different pathologies including diabetes and cardiovascular disease [94]. PCOS patients are commonly found to be insulin-resistant, irrespective of their weight or infertility, and exhibit hyperinsulinemia.

Infertility is an associated problem in patients with PCOS, directing many of the women to seek help through assisted reproductive techniques (ART). According to the World Health Organization (WHO), women with ovulatory disorders, including PCOS, can be successfully treated with ovulation induction along with timed intercourse or any other form or assisted fertilization [4]. Due to the increased risk of ovarian hyperstimulation syndrome and multiple pregnancies, intrauterine insemination (IUI) is the least favored method of ART that is adopted for PCOS patients. Instead, IVF has become the more effective option for women with PCOS to become pregnant [4].

PCOS was thought to be due to genetic predisposition, including evolutionary and environmental modifications. This in turn led to genetic studies of women with PCOS in comparison with disease-free controls but no definitive answers were obtained [95]. The lack of any conclusive information from genetic studies led scientists to focus more on the protein complements instead. The ability to identify certain molecules that are characteristically under- or over-expressed in PCOS patients compared with controls could provide additional insights into the disease. In addition to better understanding of the disease, advanced tools including proteomics will also provide physicians with an excellent tool for diagnosis of a disease that is currently difficult to diagnose, due to multiple manifestations. It would also aid in the identification of therapeutic agents and newer treatment modalities [96]. By examining the proteins, researchers may also have insight into the biological pathways involved with PCOS. This could eventually help in the identification of changes occurring due to oxidative stress or different environmental factors.

Proteomic studies for PCOS have been conducted on different body fluids and tissues including ovarian tissue, serum, visceral adipose tissue, plasma, and ovarian granulosa

cells. Follicular fluid has been utilized extensively due to its easy accessibility by transvaginal ultrasound-guided oocyte retrieval, following hormonal treatment. The vascularization of the theca interna allows the transduction of different factors from circulation transuding into the follicular fluid, thus providing a representative study medium [38]. Follicular fluid contains metabolic products of granulosa cells, proteins from the follicle and blood, hormones, growth factors, and stem cells and will thereby provide information about the function of each of these molecules in the process of folliculogenesis [14].

Dai and Lu were able to identify 20 differentially expressed proteins when comparing PCOS patients and normal women. The proteins were involved in glucose metabolism, lipoprotein metabolism, cell proliferation, apoptosis, and insulin resistance [14]. While Cortón et al. were able to identify only 15 differentially expressed proteins, Ma et al. identified 69 proteins [6, 13]. Several other studies were also able to identify differentially expressed proteins in different testing media. A list of the more common proteins is presented in Table 2.

Heat shock proteins (HSP) are low molecular weight molecules that can function at temperatures higher than other proteins. They are usually responsible for maintaining cellular homeostasis and act as chaperones of proper protein folding. According to Ma et al., HSP27 (Table 2) is highly expressed in normal ovarian tissue compared to tissue from PCOS patients. In this study, only women in their follicular phase, younger than 30 years with regular menstrual cycles, were selected as controls. Patients with PCOS were only included if they had >12 follicles in one ovary or 2 of the 4 criteria: irregular menstrual cycles, polycystic ovaries, hyperandrogenism, and chronic anovulation [13]. This protein is responsible for maintaining proper protein shape and configuration and acts as an antioxidant in cells. In stressful cellular environments, HSP27 expression was shown to be elevated in order to confer resistance against oxidative stress [97]. HSP27 was also shown to suppress apoptosis through different methods, either by inhibiting cellular caspases, deactivating the ROS in the cell, or blocking Fas-induced apoptosis. The downregulation of this protein indicated lower levels of antioxidants in PCOS ovaries, implying the involvement of oxidative stress underlying PCOS and infertility [13].

Iron is a heavy metal capable of having multiple oxidative levels and therefore acts as a redox molecule. The ability of iron to transfer electrons endows it with the power of forming free radical molecules. Free iron catalyzes the formation of hydroxyl radicals by the Fenton/Haber-Weiss reaction, requiring iron to remain bound within solutions. Transferrin is an iron-binding protein present in different biological fluids [98]. An increase in free iron is thought to cause oxidative stress within cells with concurrent increase in ROS levels. Women with PCOS are thought to exhibit more oxidative stress in follicular fluid [38]. This, in turn, would mean lower transferrin levels within follicular fluid. On the contrary, Dai and Lu found increased levels of transferrin in follicular fluid during controlled ovarian hyperstimulation [14]. It was postulated that this was due to the high levels of transferrin that inhibited FSH from binding to granulosa

TABLE 2: Proteins differentially expressed in PCOS patients.

Protein and method	Function	Site of expression	Relation to PCOS	Population size	Reference
HSP27/2D gel electrophoresis and mass spectrometry	Steroidogenesis, chaperoning and protection against apoptosis	Ovarian tissue	Decreased	3 normal women and 3 with PCOS all younger than 30 years old	[13]
HSP10/2D gel electrophoresis and mass spectrometry	Protect against apoptosis and play a role in follicular atresia	Ovarian tissue	Decreased	3 normal women and 3 with PCOS all younger than 30 years old	[13]
HSP47/2D gel electrophoresis and mass spectrometry	Collagen specific molecular chaperone involved in fibrolytic disease	Ovarian tissue	Increased	3 normal women and 3 with PCOS all younger than 30 years old	[13]
FUSE binding protein 1/2D gel electrophoresis and mass spectrometry	Regulation of insulin function	Ovarian tissue	Increased	3 normal women and 3 with PCOS all younger than 30 years old	[13]
Glyoxylate reductase/Hydroxypyruvate reductase/2D gel electrophoresis and mass spectrometry	Regulation of insulin function	Ovarian tissue	Increased	3 normal women and 3 with PCOS all younger than 30 years old	[13]
Antithrombin III/2D gel electrophoresis and mass spectrometry	Regulation of fibrinolysis and thrombosis	Ovarian tissue	Decreased	3 normal women and 3 with PCOS all younger than 30 years old	[13]
Annexin A2/2D gel electrophoresis and mass spectrometry	Regulation of fibrinolysis and thrombosis	Ovarian tissue	Decreased	3 normal women and 3 with PCOS	[13]
Fibrinogen alpha and gamma chain/2D gel electrophoresis and mass spectrometry	Fibrinolysis and blood clotting	Ovarian tissue	Increased	3 normal women and 3 with PCOS all younger than 30 years old with PCOS	[13]
Laminin A/C/2D gel electrophoresis and mass spectrometry	Regulators of the ovary	Ovarian tissue	Increased	3 normal women and 3 with PCOS all younger than 30 years old	[13]
Vimentin/2D gel electrophoresis and mass spectrometry	Regulator of the ovary	Ovarian tissue	Increased	3 normal women and 3 with PCOS all younger than 30 years old	[13]
Vitamin D binding protein (DBP)/2D gel electrophoresis and MALDI-TOF	Binding vitamin D	Follicular fluid	Decreased	30 normal women undergoing IVF and 30 with PCOS	[14]
Plasma retinol binding protein (RBP4)/2D gel electrophoresis and MALDI-TOF	Binding of retinol	Follicular fluid	Increased	30 normal women undergoing IVF and 30 with PCOS	[14]
Transferrin (TTR)/2D gel electrophoresis and MALDI-TOF	Insulin resistance and obesity	Follicular fluid	Increased	30 normal women undergoing IVF and 30 with PCOS	[14]

TABLE 2: Continued.

Protein and method	Function	Site of expression	Relation to PCOS	Population size	Reference
Apolipoprotein A1/2D gel electrophoresis and MALDI-TOF	Regulate lipid metabolism and has a function in anti-inflammatory pathways	Follicular fluid	Increased	30 normal women undergoing IVF and 30 with PCOS	[14]
Apolipoprotein A4/2D gel electrophoresis and MALDI-TOF	Weakest lipophilic capacity and associated with lipid metabolism	Follicular fluid	Increased	30 normal women undergoing IVF and 30 with PCOS	[14]
Transferrin/2D gel electrophoresis and MALDI-TOF	Binds iron and transports it. Promote cell growth and development	Follicular fluid	Increased	30 normal women undergoing IVF and 30 with PCOS	[14]
DRAM2/2D gel electrophoresis and MALDI-TOF	Apoptosis	Follicular fluid	Decreased	30 normal women undergoing IVF and 30 with PCOS	[14]
MAK (Male germ-associated kinase)/2D gel electrophoresis and MALDI-TOF	Plays a role in meiosis of cells	Follicular fluid	Decreased	30 normal women undergoing IVF and 30 with PCOS	[14]
Glutathione S-transferase M3 (GSTM3)/2D gel electrophoresis and MALDI-MS	Antioxidant involved in breakdown of cytotoxic material	Omental adipose tissue	Increased	19 obese women undergoing bariatric surgery; 10 with PCOS and 9 nonsymptomatic used as control	[6]
Annexin V/2D gel electrophoresis and MALDI-MS	Phospholipid binding	Omental adipose tissue	Increased	19 obese women undergoing bariatric surgery; 10 with PCOS and 9 nonsymptomatic used as control	[6]
Peroxisiredoxin 2/2D gel electrophoresis and MALDI-MS	Antioxidant	Omental adipose tissue	Decreased	19 obese women undergoing bariatric surgery; 10 with PCOS and 9 nonsymptomatic used as control	[6]
Actin/2D gel electrophoresis and MALDI-MS	Cytoskeletal protein and gene expression regulation	Omental adipose tissue	Decreased	19 obese women undergoing bariatric surgery; 10 with PCOS and 9 nonsymptomatic used as control	[6]
TPH1/2D gel electrophoresis and MALDI-MS	interconversion of dihydroxyacetone phosphate and glyceraldehydes-3-phosphate in preparation for glycolysis	Omental adipose tissue	Decreased	19 obese women undergoing bariatric surgery; 10 with PCOS and 9 nonsymptomatic used as control	[6]
Prolidase	Matrix metalloproteinase	Serum	Increased	30 PCOS patients and 28 normal women	[37]

cells. The diminished levels of FSH, binding to granulosa cells, result in altered follicular growth and maturation as proposed by Kawano et al. [99].

Apolipoprotein A1 (APOA1) is a component of high-density lipoprotein (HDL), proven to maintain the structure and function of the lipoprotein. Apolipoprotein A4 (APOA4) is also a member of the apolipoprotein family with the weakest lipophilic ability. Dai and Lu showed that both APOA1 and APOA4 were significantly increased in the follicular fluid of PCOS patients when compared with patients without PCOS, proving their role in lipid metabolism and abnormal ovulation. These proteins were identified by 2D-GE and MALDI-TOF and confirmed by Western blotting [14]. Lipid metabolism may eventually lead to the formation of lipid peroxides that contribute to the ROS content of a cell. It was shown that lipid peroxides are usually associated with HDL and not LDL, suggesting their association with APOA1 [100, 101]. Such indications led researchers to postulate the role of APOA1 as a biomarker for PCOS due to its presence in plasma and follicular fluid making it an easy target to assess.

Peroxiredoxin (Prx) and glutathione S-transferase M3 (GSTM3) are both antioxidant proteins that were significantly expressed in adipose tissue of PCOS patients. The relation of oxidative stress with PCOS was evident through the differential expression of these proteins between control subjects and PCOS patients in a study done by Cortón et al. The study included 19 morbidly obese women who were undergoing bariatric surgery. 10 of these women were diagnosed with PCOS. 9 women included in the study as controls exhibited no signs of hyperandrogenism and had regular menstrual cycles. The diagnosis of PCOS was based on the presence of oligo-ovulation, hyperandrogenism, and hirsutism, excluding hyperprolactinemia, congenital adrenal hyperplasia, and any androgen secreting tumors [6]. Interestingly, Prx was decreased as GSTM3 increased, although they both function as antioxidants within the cell. As the cells possess lower antioxidant capacity, due to a decrease in Prx, they become more susceptible to oxidative stress. In an attempt to correct for this imbalance, adipose cells produce more GSTM3 which explains the discrepancy between the expression levels of these two proteins. GSTM3 also functions in the breakdown of cytotoxic cellular components which, in turn, leads to an increase in oxidative stress levels in the cell [6]. Therefore, the difference in expression between the two proteins may appear to work in opposite directions when in fact over- and underexpression of each of these proteins lead to the same result: increase in oxidative stress due to decrease in antioxidant activity and the breakdown of cytotoxic cell components.

In the same study by Cortón et al., it was also noted that the expression of annexin V increased in the adipose tissue of women with PCOS [6]. Several functions have been attributed to the annexin family of proteins. They appear to function in regulation of membrane organization and may also be involved with apoptosis [102]. Leon et al. showed that annexin V in particular was involved in a series of cascade reactions that in turn led to the binding of the protein to the interferon-gamma receptor [103]. Interferon-gamma is a cytokine responsible for the production of ROS in the body

and in this case explains the increase in annexin V and its significance is demonstrated by increased levels of ROS in PCOS patients.

The extracellular matrix (ECM) and the cellular environment are critical players in PCOS pathology. The ECM is the microenvironment around cells that provides them with physical support. It is also responsible for transmitting signals for the different cellular functions [37]. Matrix metalloproteinases (MMP) are extracellular peptidases that hydrolyze several proteins including those making up the ECM and therefore could contribute to the altered cellular environment and various pathologies [104]. Prolidase is one such MMP involved in collagen remodeling and cell growth and its activity was found to be correlated with oxidative stress levels in the body [105]. Hilali et al. studied the amount of prolidase expressed in PCOS patients in an attempt to assess the association of MMP with the disease. The results showed that prolidase was increased in the serum of PCOS patients in comparison to normal women in addition to the increase in oxidative stress levels [37]. Multiple studies reported increased prolidase activity in patients with cardiovascular disease [106]. The increased degradation of the ECM can also be a causative agent of PCOS, possibly due to the remodeling of ovarian tissue structure and that could subsequently lead to the condition of polycystic ovaries. Different MMP molecules are already being used as biomarkers for the detection of different cardiovascular diseases [106]. Increased activity of prolidase is almost always accompanied with oxidative stress in the pathology of PCOS; they may be used in combination with other clinical factors as biomarkers for early and accurate diagnosis of PCOS in women where diagnosis has been difficult.

7. Role of Proteomics in Unexplained Infertility

Unexplained infertility is a diagnosis made by exclusion when no other factors seem to contribute to this pathophysiology [39]. Although no definitive cause of infertility in cases of idiopathic infertility has been established yet, several studies have linked the diagnosis of unexplained infertility with an increase in oxidative stress and the converse where there is an improvement in the fertility status following antioxidant supplementation also holds true [39]. The infertility diagnosis in women is generally established after ruling out male factor, ovulatory dysfunction, hormonal causes, and other known causes of infertility. According to the study performed by Smith et al., 30% of infertile couples are labeled as having unexplained infertility. One of the major contributing factors is usually the age of the female partner [107].

Gleicher and Barad suggested that unexplained infertility is not a diagnosis but is the lack of one and therefore impossible to prove. They argued that the use of the word “unexplained infertility” is inaccurate as it would mean that all of the reproductive workup has been done, when in fact there is no definitive answer as to what represents a complete reproductive workup [108, 109]. They go on to explain the different reasons that could cause infertility that are not usually

checked by physicians such as premature aging of ovaries, autoimmune disease, or even misdiagnosed tubal disease [109].

Endometrial receptivity is another cause of infertility that clinicians are currently unable to characterize with definitive markers [44]. The endometrium is receptive for only 4-5 days of the menstrual cycle allowing a narrow window for embryonic implantation. During this time, the cells become secretory and release several molecules that help in implantation and embryo development in what is called the window of implantation [110]. It is important that physicians are able to identify such molecules in the uterine fluid and tissue, within that time frame, in order to provide a noninvasive method to check for the appropriate environment for establishing a pregnancy. Implantation failure is currently the most common limiting factor in achieving pregnancy in women undergoing ART. Therefore, establishing endometrial receptivity is crucial for improved outcomes with ART [111]. Several reports indicate that genes expressed at different stages of the endometrial cycle do not adequately represent the protein expression during that time and hence the need for proteomics for the identification of potential biomarkers of endometrial receptivity [112]. Some of the proteins identified are listed in Table 3.

In the experiment performed by Domínguez, both annexin A2 and S100-A10, components of the annexin heterotetramer (A11t), were upregulated in the endometrial tissue obtained during the prereceptive and receptive phase of the menstrual cycle. These protein measurements were conducted by differential in-gel electrophoresis and MALDI-MS, during the endometrial receptive phase. These proteins were shown to be associated with higher chances of implantation [113]. Annexin A2 was originally thought to function in cell-cell interaction and adhesion. However, different biochemical alterations, such as glycosylation and glutathionylation, may cause functional alterations secondary to changes in the structure. Glutathionylation seems to occur with development of oxidative stress. The changes incurred on a protein due to either glutathionylation or glycosylation may be inhibitory or stimulatory. Glutathionylation of A11t causes a decrease in the protein's biological function. Both annexin A2 and S100-A10 activity were reduced in cases with higher levels of oxidative stress [120]. A decrease in annexin concentration could in turn inhibit cellular reorganization in endometrial cells. The lack of annexin A2 during the receptive phase is one example where oxidative stress may alter protein function and structure leading to detrimental effects at the cellular level. Without the remodeling of endometrial cells and cell-to-cell dialogue, pregnancy outcomes decrease with disruption of implantation.

8. Proteomics and ART

The promise of assisted reproductive techniques to achieve pregnancy has led numerous infertile couples to opt for the procedures, with the hope of fulfilling their dream of having their own biological child. It is, however, noted that these techniques are not always associated with higher clinical

pregnancy and live birth rates. To achieve a high pregnancy rate it is important to understand the proteins involved in follicular microenvironment [117]. Women have to undergo controlled ovarian stimulation to isolate mature oocytes for the use in IVF or ICSI procedures. In an ART setting, the effects of oxidative stress are amplified due to the lack of internal defense mechanisms that would normally operate *in vivo* as well as increased levels of ROS originating as a result of manipulation of male and female gametes [121]. The high ROS levels may impact the energy pathways in the newly formed embryo and impair embryonic development [121].

Oxidative stress levels were studied by Younis et al. in women undergoing ovarian stimulation prior to ART. In the study by Younis et al., it was noted that the level of antioxidant proteins increased during the peak estrogen levels [9]. The increase in superoxide dismutase (SOD), paraoxonase 1 (PON1), and interleukin 6 (IL6) in plasma correlated positively with pregnancy outcomes and was therefore thought to have potential use as biomarkers for predicting positive pregnancy (Table 3) [9]. Prieto et al. showed that the increase in SOD levels in follicular fluid was predictive of successful pregnancies in IVF patients; hence it could potentially be utilized as a biomarker [31]. Both PON1 and SOD are enzymatic antioxidants present in the blood and their upregulation during ovarian stimulation highlights the role of oxidative stress in reducing pregnancy rates. However, this is in contrast to other studies that showed a higher amount of oxidative stress during ovarian stimulation with a concomitant reduction in antioxidant proteins [100].

Jarkovska et al. conducted a study in which they compared the protein expression within follicular fluid and plasma in women undergoing ovarian stimulation. It was reported that the immunological pathways are one of the major determinants of successful IVF rates. A regulated complement system seems to be essential for proper implantation and embryo maturation during pregnancy with successful IVF procedures. The activity of the complement system decreased VEGF activity leading to less blood vessel formation within the placenta. The inappropriate complement activity is also directly related to a higher rate of abortions. In addition, proteins involved with angiogenesis and blood coagulation seem to be essential in achieving a successful pregnancy following IVF. The list of proteins identified for optimal embryo implantation included antithrombin III precursor, complement precursors, and fibrinogen beta chain precursor (Table 3) [117]. As noted earlier, VEGF levels increase in response to higher levels of oxidative stress further implicating that ROS has a negative effect on ART success rates [72].

The levels of complement C3 and antithrombin were decreased in the follicular fluid in a group of young women who had successful live births in comparison with those who underwent IVF procedures with no success. In addition, higher levels of fibrinogen alpha chain were associated with increased likelihood of live births [122]. Fibrinogen levels in the peritoneal fluid were previously highlighted in this review to serve as potential biomarkers for the presence of endometriosis [47]. Higher levels of fibrinogen may be an indicator of the hypercoagulable state of pregnancy as higher levels

TABLE 3: Proteins identified at different endometrial stages, in IVF and in the embryo.

Protein estimated/proteomic technique	Function	Source	Relation to endometrial receptivity or embryo readiness	Population size	Reference
L-Glutamate NMCA receptor zeta subunit 1/LC-MS/MS	Subunit of a ligand gated channel that is involved in neuron plasticity. Other roles include glutamate mediated toxicity in mitochondria leading to apoptosis	Endometrial tissue	Only expressed in the secretory endometrium	6 samples of endometrial tissue from a tissue bank; 3 from proliferative phase and 3 from secretory phase (as classified by a pathologist)	[46]
FRAT1/LC-MS/MS	Proto-oncogene that activates the WNT pathway. Inhibits apoptosis	Endometrial tissue	Only expressed in the secretory endometrium	6 samples of endometrial tissue from a tissue bank; 3 from proliferative phase and 3 from secretory phase (as classified by a pathologist)	[46]
Myosin light chain kinase 2/LC-MS/MS	Muscle contraction	Endometrial tissue	Only expressed in the secretory endometrium	6 samples of endometrial tissue from a tissue bank; 3 from proliferative phase and 3 from secretory phase (as classified by a pathologist)	[46]
Isopentenyl diphosphate delta-isomerase/LC-MS/MS	Catalyzes a step in the formation of isoprenoids.	Endometrial tissue	Only expressed in the secretory endometrium	6 samples of endometrial tissue from a tissue bank; 3 from proliferative phase and 3 from secretory phase (as classified by a pathologist)	[46]
Macrophage migration inhibitor factor (MIF)/LC-MS/MS	Prevent macrophages from performing their job in the endometrium	Endometrial tissue	Found in all phases of the endometrium	6 samples of endometrial tissue from a tissue bank; 3 from proliferative phase and 3 from secretory phase (as classified by a pathologist)	[46]
Glycodelin/LC-MS/MS	Regulating the uterine cavity for pregnancy	Endometrial tissue	Up-regulated during the secretory phase	6 samples of endometrial tissue from a tissue bank; 3 from proliferative phase and 3 from secretory phase (as classified by a pathologist)	[46]
Stathmin 1/gel electrophoresis and MALDI-MS	Cytoskeleton, intracellular signaling cascade	Endometrial tissue	Decreased during receptive phase	8 fertile women in their reproductive age, during pre-receptive and receptive times of the menstrual cycle	[113]
Annexin A2/gel electrophoresis and MALDI-MS	Skeletal development		Increased during receptive phase	8 fertile women, in their reproductive age, during pre-receptive and receptive times of the menstrual cycle	[113]
Monoamine oxidase A/gel electrophoresis and MALDI-MS	Electron transport	Endometrial tissue	Increased during receptive phase	8 fertile women in their reproductive age, during pre-receptive and receptive times of the menstrual cycle	[113]
Membrane-associated progesterone receptor component 1/gel electrophoresis and MALDI-MS	Signaling	Endometrial tissue	Decreased during receptive phase	8 fertile women in their reproductive age, during pre-receptive and receptive times of the menstrual cycle	[113]

TABLE 3: Continued.

Protein estimated/proteomic technique	Function	Source	Relation to endometrial receptivity or embryo readiness	Population size	Reference
Collagen alpha 1/gel electrophoresis and MALDI-MS	Skeletal development	Endometrial tissue	Increased during receptive phase	8 fertile women, in their reproductive age, during pre-receptive and receptive times of the menstrual cycle	[113]
Annexin A4/gel electrophoresis and MALDI-MS	Signal transduction	Endometrial tissue	Increased during receptive phase	8 fertile women in their reproductive age, during pre-receptive and receptive times of the menstrual cycle	[113]
Vimentin/gel electrophoresis and MALDI-MS	Cytoskeleton	Endometrial tissue	Decreased during receptive phase	8 fertile women, in their reproductive age, during pre-receptive and receptive times of the menstrual cycle	[113]
SI00-A10/gel electrophoresis and MALDI-MS	Signal transduction	Endometrial tissue	Increased during receptive phase	8 fertile women in their reproductive age, during pre-receptive and receptive times of the menstrual cycle	[113]
Sulfur oxide dismutase	Enzymatic antioxidant	Serum	Increased during ovarian stimulation	15 patients undergoing ovarian stimulation who had PCOS, endometriosis, or unexplained infertility and all went controlled ovarian stimulation	[9]
Paraoxonase 1 (PON1)	Enzymatic antioxidant	Serum	Increased during ovarian stimulation	15 patients undergoing ovarian stimulation who had PCOS, endometriosis, or unexplained infertility and all went controlled ovarian stimulation	[9]
Glutathione peroxidase (GPx)	Enzymatic antioxidant	Serum	Increased during ovarian stimulation	15 patients undergoing ovarian stimulation who had PCOS, endometriosis, or unexplained infertility and all went controlled ovarian stimulation	[9]
Interleukin 6	Acts as both an anti-inflammatory and proinflammatory cytokine	Serum	Increased during ovarian stimulation	15 patients undergoing ovarian stimulation who had PCOS, endometriosis, or unexplained infertility and all went controlled ovarian stimulation	[9]
Apolipoprotein AI/Tandem MS	Component of high density lipoproteins and acceptor of extra-hepatic cholesterol	Embryo culture media	Decreased in secretome of embryos	702 samples analyzed from women undergoing IVF or from egg donor recipients irrespective of age (26–49 years) or reason for infertility	[11]
Leptin/RT-PCR	Regulates energy uptake and expenditure	Endometrial culture	Increased in competent embryos	Embryos and granulosa cells from 9 patients after ovarian hyperstimulation and artificial insemination. Human adipose tissue was used as control for leptin levels. Human endometrial and placental tissue was also studied	[114]
HOXA10/RT-PCR	Functions in embryo viability	Ishikawa cells which are well-differentiated endometrial adenocarcinoma cell line	Increased in cells exposed to blastocyst media	Ishikawa cells grown with blastocyst cells and ones that were placed with an embryo not yet in the blastocyst stage	[115]

TABLE 3: Continued.

Protein estimated/proteomic technique	Function	Source	Relation to endometrial receptivity or embryo readiness	Population size	Reference
Lipocalin-1/mass spectrometry	Transport proteins	Blastocyst secretions	Used for non-invasive aneuploidy testing	65 couples undergoing infertility treatment. Samples were pooled for MS into a group that had 100% implantation from euploid blastocysts or media of aneuploid blastocysts. IVF media without an embryo were used as control	[116]
Complement precursor proteins/2D gel electrophoresis, 2D HPLC and MALDI-MS	Part of the innate immune system	Human follicular fluid and plasma	Altered expression during IVF procedures	38 women (24–38 years) undergoing stimulation prior to IVF. All the samples were used from women with 100% success of IVF	[117]
Fibrinogen Beta precursor/2D gel electrophoresis, 2D HPLC and MALDI-MS	Involved in blood clotting cascade	Human follicular fluid and plasma	Altered expression during IVF procedures	38 women (24–38 years) undergoing stimulation prior to IVF. All the samples were used from women with 100% success of IVF	[117]
Antithrombin III precursor/2D gel electrophoresis, 2D HPLC and MALDI-MS	Inactivates the coagulation system	Human follicular fluid and plasma	Altered expression during IVF procedures	38 women (24–38 years) undergoing stimulation prior to IVF. All the samples were used from women with 100% success of IVF	[117]
Heparin-binding epidermal growth factor- (EGF-) like growth factor precursor/SELDI-TOF-MS	Cell-to-cell juxtacrine signaling inhibiting growth activity	Human embryonic cells	Increased in embryos that failed to develop	21 blastocysts at different developmental stages that had been cryopreserved. They were graded as early, expanded, or degenerated embryos	[118]
Cystatin like precursor/SELDI-TOF-MS	Inhibits cysteine proteases involved in implantation	Human embryonic cells	Increased in embryos that failed to develop	21 blastocysts at different developmental stages that had been cryopreserved. They were graded as early, expanded, or degenerated embryos	[118]
Caspase 1 precursor/SELDI-TOF-MS	Involved in cell death	Human embryonic cells	Increased in embryos that failed to develop	21 blastocysts at different developmental stages that had been cryopreserved. They were graded as early, expanded, or degenerated embryos	[118]
Cytochrome c oxidase subunit VIIIa 3/SELDI-TOF-MS	Part of the electron transport chain in mitochondria	Human embryonic cells	Increased in embryos that failed to develop	21 blastocysts at different developmental stages that had been cryopreserved. They were graded as early, expanded, or degenerated embryos	[118]
Ubiquitin/SELDI-TOF-MS	Part of the ubiquitin-proteasome pathway involved in degradation of proteins	Mice and human embryo secretome	Increased in embryonic secretome associated with successful pregnancies	2-cell human embryos and mouse embryos. Mouse embryos were collected after hyperstimulation of mouse and fertilization. Human embryos were obtained from cryopreserved embryos	[119]

of fibrinogen have been reported along with a decrease in fibrinolysis [123]. The altered expression of these various proteins and their successful reproducibility in multiple experiments reinforce their potential role as a diagnostic tool in infertility and ART.

9. Protein Profiles of Embryos in ART

In addition to its implementation in analyzing success rates of IVE, proteomics has been used to study the secretome of embryos in the hope of discovering a biomarker to assess and predict embryo viability. However, the knowledge on preimplantation embryo is currently limited due to technical difficulties. Restrictions such as limited template of known proteins, low quantity, and lack of sensitivity of equipment are all impediments that should be overcome before definitive studies can be accomplished [15]. Embryo selection in ART is currently being done using morphology as the only determinant of embryo viability. Such methods present with several limitations and most importantly, its lack of representation of genomic differences [4]. Several studies have looked at the protein profile of embryos and their secretome in order to determine a suitable marker for implantation success. Some of the proteins identified are listed in Table 3.

Katz-Jaffe et al. identified multiple differentially expressed proteins between blastocysts that matured or did not and blastocysts at different developmental stages as well as embryos that were morphologically similar but did not necessarily possess the same protein profile (listed in Table 3) [118]. Embryos that failed to develop were shown to have higher levels of apoptotic and growth inhibiting proteins including cystatin-like precursor protein which functioned in inhibiting cysteine proteases [118]. Protease activity is crucial in the degradation of the extracellular matrix in the uterine environment and therefore in implantation [124]. The high expression level of cystatin-like precursor protein can function as a potential biomarker for successful embryo implantation and help select the embryos with the highest chance of implantation. Although not proven in embryos, studies on rat CNS neurons have shown that an increase in oxidative stress eventually leads to an increase in cystatin C activity. This may therefore explain why an increase in oxidative stress *in utero* is linked to lower fertility status [125].

Katz-Jaffe et al. found that the proteins secreted from the embryo over a period of 24 hours were highly reflective of what was going on inside the cells, as proteins are the major determinants of cell function [119]. Ubiquitin, a polypeptide that functions as part of the protein degrading complex, was found to be differentially expressed in different cells. Significantly higher levels of the protein were observed in developing blastocyst secretome but seemed to be diminished in the secretome of degenerating embryos [119].

The ubiquitin-proteasome pathway (UPP) was shown to be involved in uterine remodeling and placental development which allows successful implantation. Wang et al. were able to prove that blocking this pathway inhibited pregnancy in different species including humans [126]. The change of ubiquitin expression in the uterine tissue also altered the activity

of the matrix metalloproteinases which are important during the early pregnancy [126, 127]. Ubiquitin levels measured in the secretome of the embryo may therefore be used as a biomarker for noninvasive and easy prediction of successful pregnancy.

Proteomic studies on the embryo are still limited due to experimental difficulties especially in human embryos. Finding a greater number of differentially expressed proteins will increase our knowledge on the activity of embryos and what is essential for successful implantation and pregnancy. These proteins may also be used as biomarkers to select the embryos before transferring them during ART. This can help avoid unsuccessful transfers as well as the transfer of karyotypically abnormal embryos.

10. Conclusion

In this review, we have highlighted studies that have identified some of the proteins in female infertility-related diseases and their association with underlying oxidative stress. Endometriosis, PCOS, and unexplained infertility are currently the three most common causes of infertility among women worldwide. Understanding of these diseases will provide clinicians with a better insight into their early diagnosis and management. Identification of proteins in different diseases and at different stages of the progression will help in the discovery of potential biomarkers for noninvasive diagnosis of these diseases. The various proteins listed in the review have been separated, identified, and confirmed using 2D-GE, mass spectrometry, and Western blotting, respectively. 2D-PAGE is beneficial in its ability to detect proteins with wide range of masses and identify several proteins at any one time. However, it is a time-consuming process and requires extensive laboratory skills. Mass spectrometry has the advantage of being rapid and can utilize even a small sample size but is limited by the availability of test samples that can be used. Western blotting is characterized by its high sensitivity and specificity; however, some drawbacks in the technique include its high technical demand and being prone to false results. In addition, we looked at proteins identified in the embryonic secretome. Definitive knowledge of the embryonic secretome can assist in the selection of the best embryo and help clinics to move towards single embryo transfers without any impact on the success rates.

Future research should be directed to identify specific proteins in the abovementioned diseases as well as proteins related to identification of best embryos. Researchers should also aim to achieve reproducible results and validate these proteins as this is a main requirement in biomarker credibility. The ability to identify a protein, common between women of different races, ages, and in different regions of the world, will hopefully contribute to improving women's health worldwide.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Association between Follicular Fluid Leptin and Serum Insulin Levels in Nonoverweight Women with Polycystic Ovary Syndrome

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Aims. We evaluated the links between leptin and visfatin levels and fertilization rates in nonoverweight (NOW) women with PCOS (NOW-PCOS) from Apulia undergoing in vitro fertilization/embryo transfer (IVF). **Materials and Methodology.** We recruited 16 NOW women with PCOS (NOW-PCOS) and 10 normally ovulating NOW women (control-NOW). All women underwent IVF. Androgens, 17- β -estradiol (17 β -E₂), and insulin levels were measured in plasma and/or serum and leptin and visfatin levels were assayed in both serum and follicular fluid (FF-leptin, FF-visfatin). **Results.** In NOW-PCOS, both serum and FF-leptin were significantly lower than in control-NOW. In NOW-PCOS, significant correlations were found between BMI and serum leptin and insulinemia and FF-leptin. By contrast, in control-NOW, FF-leptin levels were not correlated with insulinemia. Serum visfatin levels were not significantly different in NOW-PCOS and control-NOW, but FF-visfatin levels were 1.6-fold higher, although not significantly, in NOW-PCOS than in control-NOW. **Conclusions.** Both serum leptin levels and FF-leptin are BMI- and insulin-related in Southern Italian NOW-PCOS from Apulia. In line with other reports showing that FF-leptin levels are predictive of fertilization rates, lower than normal FF-leptin levels in NOW-PCOS may explain their lower fertilization rate and this may be related to the level of insulin and/or insulin resistance.

1. Background

Polycystic ovary syndrome (PCOS) is commonly defined, according to the Amsterdam ESHRE/ASRM PCOS Consensus Workshop Group [1], as a disorder including anovulation, menstrual disturbances, and hyperandrogenism and is often associated with clinical features of the metabolic syndrome with an increased risk of type 2 diabetes. PCOS is the major cause of infertility in women in the fertile age. Studies on different ethnic groups support a familial and genetic predisposition to PCOS, and oligogenic/polygenic mechanisms might be involved in its etiology [2, 3]. Both hyperinsulinemia and/or insulin resistance and hyperandrogenism play an

important role in its pathogenesis [4]. In addition, obesity is often, but not always, a fellow traveller of PCOS.

In *ob/ob* mice, displaying a phenotype similar to that of human obesity, a point mutation of the *ob* gene encoding for leptin, accounts for hyperphagia, morbid obesity, and sterility [5, 6]. Interestingly, humans that are homozygous for a point mutation of the *ob* gene also display a phenotype whose main features are morbid obesity and sterility [7, 8]. Impaired function and/or reduced levels of leptin might thus cause infertility in women with PCOS. Consistent with this concept, circulating leptin levels and leptin mRNA expression in adipocytes are reduced in women with PCOS [9, 10].

Several studies have investigated leptin levels in overweight and/or obese women with PCOS, clearly demonstrating that they appear to be correlated with BMI [11]. However, granulosa cells are able to synthesize and store leptin from newborn until adult life in a manner that is independent of BMI [12]. Relevant to this concept, few studies have investigated leptin levels in normal-weight women with PCOS in specific ethnic groups in the absence of excess BMI. In an Indian study, circulating leptin levels were found to be 5-fold higher in normal-weight women with PCOS as compared with controls [13]. In a Chinese study, PCOS women were characterized by higher leptin levels both in serum and follicular fluid than controls, although the BMI of the PCOS women included in this study ranged from normal-weight to overweight and obesity [14]. The above-mentioned data are not in line with other results in Caucasian women demonstrating that the concentration of leptin in the follicular fluid (FF-leptin) seems to be predictive of the fertilization rate [15]. Fertilization rate is usually lower in PCOS women as compared with normally ovulating women. The paper by De Placido et al. [15] is the only one to find a direct correlation between the concentration of leptin in the follicular fluid (FF-leptin) and the fertilization rate. Interestingly the population studied by De Placido et al. was coming from the south of Italy and certainly showed different lifestyle and diet as compared with Indians and Chinese women.

In other recent studies, elevated circulating levels of leptin and/or increased leptin/adiponectin ratios were reported in overweight PCOS women, but no information on leptin levels in lean women with PCOS was provided [16, 17].

Visfatin acts as a cytokine but also as an enzyme and is involved in metabolic and immunological diseases. Visfatin mRNA was recently detected in human follicles, and visfatin administration was shown to improve ovarian response in conditions of superovulation in animal models [18, 19].

In this study, we have measured circulating leptin and visfatin levels and FF-leptin and FF-visfatin in a selected group of Caucasian Southern Italian nonoverweight women with PCOS (NOW-PCOS) undergoing in vitro fertilization/embryo transfer (IVF) in order to better understand the interrelationships, if any, between both cytokines and fertilization rate.

Some other papers analyzed leptin levels in both serum and follicular fluid in women having PCOS; however, very few papers included only Caucasian nonoverweight women ($\text{BMI} \leq 24.9 \text{ Kg/m}^2$) with PCOS as we did. Our pilot study is also new in the fact that all women studied were coming from Apulia, a region from the south of Italy which is very well known for Mediterranean diet.

2. Materials and Methods

In this observational pilot study, we selected 16 consecutive NOW-PCOS and 10 consecutive age- and BMI-matched normal-ovulating nonhirsute premenopausal infertile Caucasian women (control-NOW) requesting a procedure of assisted reproduction at the Centre of Pathophysiology of

Human Reproduction and Gametes Cryopreservation of the University of Bari. All women were from Apulia, in Southern Italy, and did not show any sign of metabolic syndrome. In the control-NOW group, infertility was due either to male infertility or tubal factors. The diagnosis of PCOS was based on the criteria indicated in the Amsterdam ESHRE/ASRM-Sponsored 3rd PCOS Consensus Workshop Group [1, 20]. All women with PCOS had hirsutism, oligomenorrhea, and clear diagnosis of PCOS by ultrasounds. Both NOW-PCOS and control-NOW were euthyroid and were undergoing IVF for the first time. All women underwent IVF according to a previously reported protocol [21]. Infertile women with a diagnosis of diabetes mellitus were excluded from the study to rule out potential confounding effects of hyperglycemia on leptin and fertilization rates. Indeed, all subjects had fasting glucose levels $<126 \text{ mg/dL}$ (7 mmol/L) and underwent an oral glucose tolerance test according to the American Diabetes Association guidelines [22], displaying plasma glucose levels $<140 \text{ mg/dL}$ (7.8 mmol/L) after 2 h. All women were in the premenopausal stage, as confirmed by circulating FSH and LH levels measured between day 3 and day 5 of the menstrual cycle. All women underwent IVF according to a previously reported protocol [21]. Briefly, daily GnRH-a administration (Decapeptyl 0.1 mg/mL -Ipsen S.p.A., s.c.) was started during the middle-luteal phase of the cycle before ovarian hyperstimulation until the day of human chorionic gonadotropin (hCG) administration. Ovarian hyperstimulation was initiated once the serum estradiol (E_2) level was $<30 \text{ pg/mL}$ with $150\text{--}225 \text{ IU}$ of rFSH (alpha follitropin-Gonal-F, Merck Serono S.p.A.). Dosing was individualized depending on the patient's response. When at least 3 follicles of 18 mm in diameter and a serum E_2 level of $>200 \text{ pg/mL}$ per follicle were noted, the patient was given $250 \mu\text{g}$ of choriogonadotropin alpha hormone (Ovitrelle, Merck Serono S.p.A.). Monitoring was accomplished with serial vaginal ultrasound examinations and serum E_2 level determinations. Transvaginal oocyte retrieval was performed 35 hours after hCG injection. After retrieval, cumulus cells (COCs) were removed with a 30-second exposure to HEPES-buffed medium containing 40 IU/mL hyaluronidase (SAGE, USA), and denuded oocytes were then assessed for nuclear status: those that were observed to have released the first polar body were considered mature (MII) and used for ICSI procedure [23].

Fertilization was assessed 18 hours after ICSI. Normal fertilization was confirmed when 2 clearly distinct pronuclei were present. Fertilization rate was defined as the percentage of pronucleate oocytes to the number of oocytes injected. Embryo quality was evaluated under inverted microscope on the second day of development according to Veeck [24]. A maximum of three embryos were transferred on day 2 or 3 of embryo culture under ultrasound guidance. For luteal phase support all patients received a daily dose of 400 mg of vaginal micronized progesterone (Progeffik, Effik Italia). Serum $\beta\text{-hCG}$ concentration was measured 14 days after oocyte retrieval to verify pregnancy. Clinical pregnancy was judged by observation of the gestational sac on vaginal ultrasonography after 6-7 weeks of gestation.

TABLE 1: Anthropometric and metabolic characteristics and hormone levels in the experimental subjects.

	NOW-PCOS	Control-NOW	<i>p</i> value
Age (years)	32.09 ± 3.60	34.35 ± 3.13	0.04
BMI (Kg/m ²)	22.55 ± 2.61	22.55 ± 2.55	0.99
Insulinemia (μU/mL)	10.30 ± 7.44	9.39 ± 9.27	0.73
HOMA-IR	2.57 ± 2.06	2.37 ± 2.72	0.76
FSH (mIU/mL)	5.71 ± 2.02	6.96 ± 2.34	0.07
LH (mIU/mL)	8.12 ± 4	4.77 ± 1.61	0.001
17β-E ₂ (basal) (pg/mL)	32.6 ± 13.40	95.56 ± 131.63	0.07
FF-leptin (ng/mL)	7.12 ± 1.12	14.44 ± 4.67	0.037
Serum leptin (ng/mL)	5.31 ± 1.13	11.49 ± 4.21	0.045
FF-visfatin (ng/mL)	4.55 ± 0.59	2.88 ± 0.88	0.075
Serum visfatin (ng/mL)	8.66 ± 0.81	7.24 ± 1.48	0.74

NOW: nonoverweight; control-NOW: controls; BMI: body mass index; E₂: estradiol; FF: follicular fluid; FSH: follicle stimulating hormone; LH: luteinizing hormone. Data are expressed as mean ± SEM. Differences between groups were considered significant for $p \leq 0.05$.

Follicular fluid (FF) samples were obtained during IVF treatment at the time of oocyte aspiration. Bloodless aspirates from different follicles containing mature oocytes of one patient were pooled and centrifuged (1,200 rpm for 5 min) (for 10 min at 2,000 g). The supernatants were divided in aliquots and stored in sterile tubes and stored at -80°C .

Blood samples for metabolic and hormonal evaluations were collected before the IVF stimulation protocol. Total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides circulating levels were measured with specific Dimension clinical chemistry systems (Siemens Healthcare Diagnostics Ltd.). Androgens (free and total testosterone, $\Delta 4$ -androstenedione, SHBG, DHT, DHEA, and DHEAS) were measured between day 3 and day 5 of the menstrual cycle. 17β-E₂ and insulin levels were measured in plasma and/or serum and leptin and visfatin levels in both serum and follicular fluid (FF) (DIASource Leptin-EASIA Kit, Visfatin-EASIA Kit).

The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated, as previously reported [25, 26].

The fertilization rates were calculated and correlated with the anthropometric and hormonal parameters.

All results are expressed as mean ± SEM. Comparison of continuous variables among groups was performed with paired Student's *t*-test. All statistical calculations were performed with NCSS2004 software (Kaysville, UT, USA). Statistical tests were conducted as an alpha level of 0.05 [27, 28]. A stepwise regression analysis was performed to identify significant links between anthropometric and metabolic variables and between the above mentioned variables and fertilization rates.

3. Results

In this study, we have investigated NOW-PCOS, who requested a procedure of assisted reproduction, with no signs of metabolic syndrome. Both NOW-PCOS and control-NOW had total cholesterol, HDL-cholesterol, LDL-cholesterol, and

triglycerides levels as well as arterial blood pressure levels in the normal range and showed a normal glucose tolerance (data not shown).

NOW-PCOS and control-NOW had comparable fasting glucose and insulin levels, and HOMA-IR was also not significantly different in the two groups (Table 1). As far as circulating insulin levels are concerned they were significantly correlated with BMI in both NOW-PCOS and control-NOW (Figures 1(a) and 1(b)).

In both NOW-PCOS and control-NOW, serum leptin levels positively correlated with FF-leptin levels (Figures 2(a) and 2(b)). A positive linear correlation between FF-leptin and circulating insulin levels was found in NOW-PCOS but not in control-NOW (Figures 3(a) and 3(b)).

In our cohort of NOW-PCOS, a significant positive correlation was found between BMI and serum leptin ($R^2 = 0.3838$; $r = 0.6195$; $p = 0.0181$) and a positive, although non-significant, correlation was also observed between BMI and FF-leptin ($R^2 = 0.2379$, $p = 0.0553$).

As far as visfatin is concerned, serum visfatin levels were not different in NOW-PCOS and control-NOW, but FF-visfatin levels were 1.6-fold higher, even though not significantly, in NOW-PCOS as compared with control-NOW ($p = 0.075$, Table 1). No correlation was found between FF-visfatin and circulating insulin, HOMA-IR, and FF-leptin in either NOW-PCOS or control-NOW.

Basal nonstimulated FSH levels were not significantly different between the two groups, but basal nonstimulated LH levels were significantly higher in NOW-PCOS than control-NOW ($p = 0.001$, Table 1), and basal 17β-E₂ levels were 2.9-fold lower in NOW-PCOS as compared to control-NOW, even though this difference was not statistically significant ($p = 0.07$) (Table 1). In regard to the IVF stimulation procedure, the number of total oocytes was significantly higher ($p = 0.001$) but in-vitro-matured metaphase II oocytes were lower, although not significantly ($p = 0.07$), in NOW-PCOS than in control-NOW (Table 2). Even if fertilization rate has been known to be lower in PCOS women as compared with normally ovulating women, due to IVE, the fertilization rate

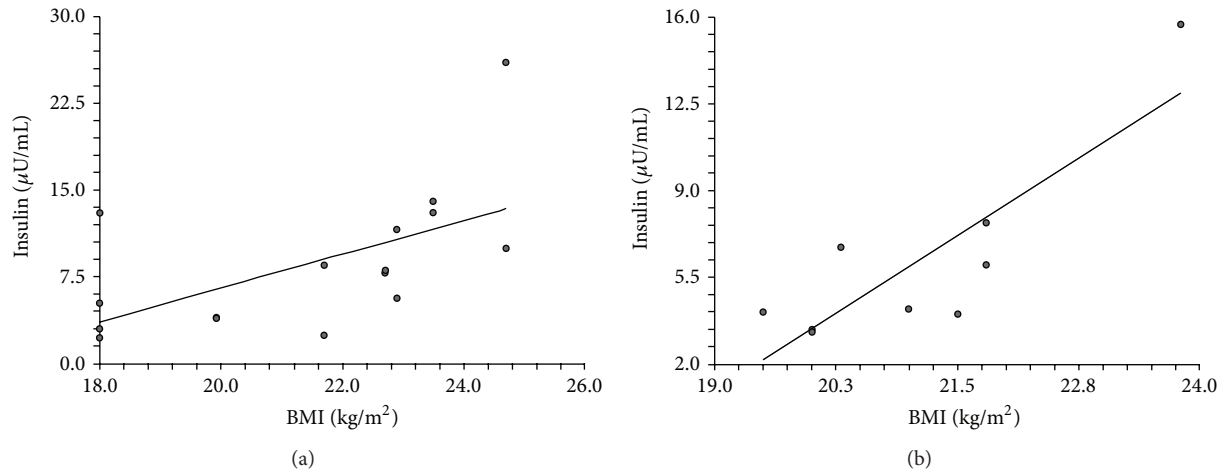


FIGURE 1: Correlation between circulating insulin levels and BMI in NOW-PCOS and control-NOW. (a) Positive linear correlation between circulating insulin levels and BMI in NOW-PCOS. $R^2 = 0.3385$; $r = 0.5818$; $p = 0.0181$. (b) Positive linear correlation between circulating insulin levels and BMI in control NOW. $R^2 = 0.7169$; $r = 0.8467$; $p = 0.0040$.

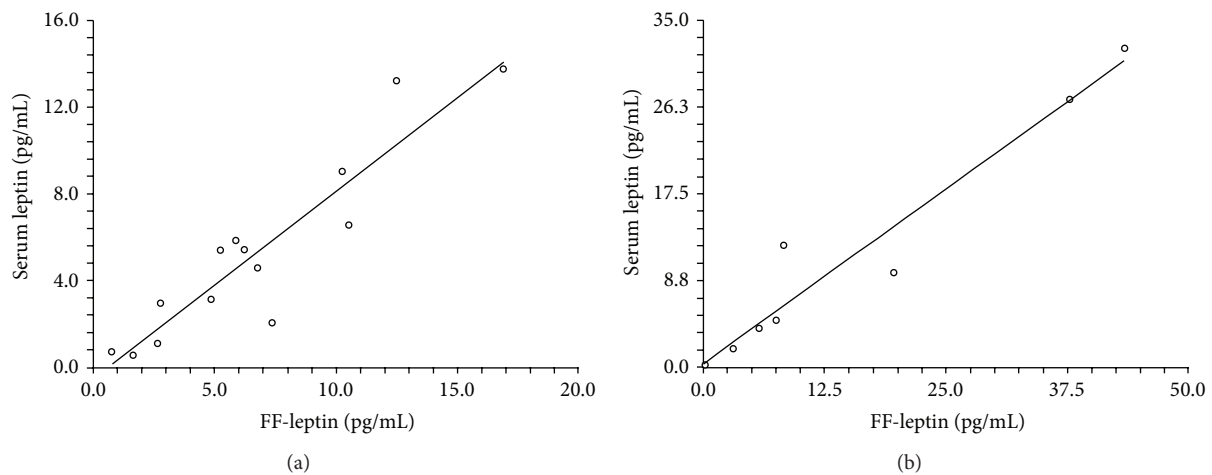


FIGURE 2: Correlation between leptin levels in the follicular fluid and circulating leptin levels in NOW-PCOS and control-NOW. (a) Positive linear correlation between leptin levels in the follicular fluid and circulating leptin levels in NOW-PCOS. $R^2 = 0.8517$; $r = 0.9229$; $p = 0.00001$. (b) Positive linear correlation between leptin levels in the follicular fluid and circulating leptin levels in control-NOW. $R^2 = 0.9376$; $r = 0.9683$; $p = 0.0001$.

resulted to be not different in the two groups. At the end of the IVF protocol, estrogen levels were threefold higher in NOW-PCOS than in control-NOW ($p = 0.001$).

4. Discussion

PCOS is highly prevalent (about 4–7%) among women in the reproductive age [25, 26] and recognizes in the impaired hypothalamus-pituitary-ovarian axis dysfunctions and hyperinsulinemia major causes driving the infertile phenotype. Nonetheless, overweight and obesity are often, but not always, associated with PCOS, and adipokines, including

leptin and visfatin, might also be involved in the pathogenesis of this disease [18–20, 29–34].

Some previous reports suggest that adipokines might be involved in the pathogenesis of PCOS [18, 31]. Since, in both animal and human models of leptin deficiency, infertility is present together with the obesity phenotype [5–8], it is to be expected that altered levels and/or function of leptin might be involved in causing infertility in overweight women with PCOS too. Leptin levels usually correlate with body fat mass, and since PCOS is often associated with overweight, the latter might represent a confounding factor in the effort to understand the role of leptin in the pathogenesis of PCOS. In this study, we investigated strictly nonoverweight women requesting a procedure of assisted reproduction and excluded

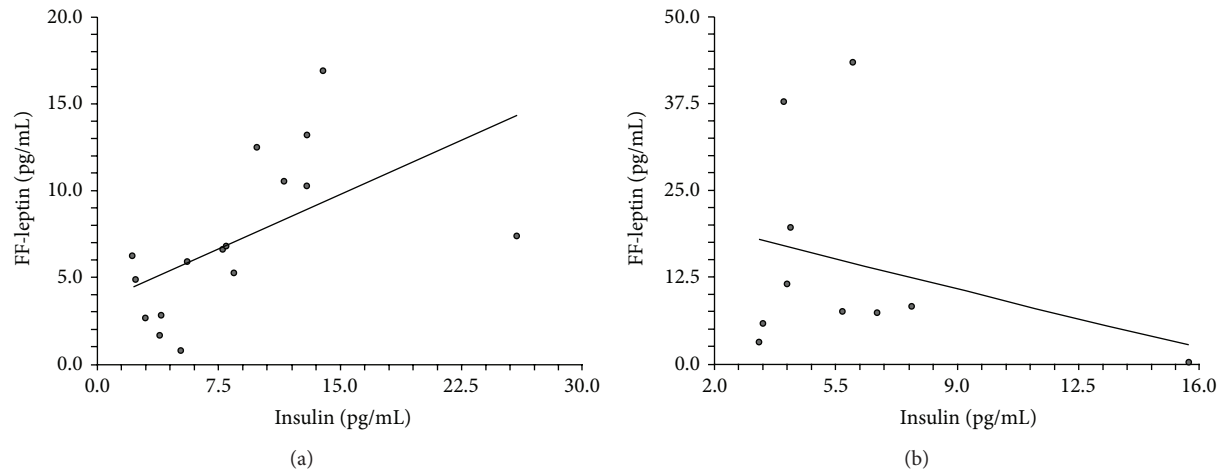


FIGURE 3: Correlation between leptin levels in the follicular fluid and circulating insulin levels in NOW-PCOS and control-NOW. (a) Positive linear correlation between leptin levels in the follicular fluid (FF) and circulating insulin levels in NOW-PCOS. $R^2 = 0.3173$; $r = 0.5633$; $p = 0.0231$. (b) Absence of correlation between leptin levels in FF and circulating insulin levels in control-NOW. $R^2 = 0.0919$; $r = -0.3032$; $p = 0.3944$.

both overweight and obese women to rule out potential confounding effects of overweight and obesity on leptin and/or visfatin levels and fertilization rates. We studied a small group of homogeneous Italian-Caucasian women from Apulia. We found significantly lower serum and FF-leptin levels in NOW-PCOS compared to BMI-matched infertile women and a positive correlation between insulinemia and FF-leptin levels in NOW-PCOS only.

In studies performed in non-Caucasian ethnic groups (Chinese, Indian), higher levels of leptin in both serum and FF were reported in PCOS women than in controls; however, in these studies the experimental groups included normal-weight, overweight, and obese PCOS women (range 19.81–30.62 Kg/m²) [13, 14]. In another recent study on Caucasian women with PCOS, elevated circulating levels of leptin and/or increased leptin/adiponectin ratios were found in overweight PCOS women, but leptin levels in lean women with PCOS were not investigated [16].

Nonetheless, results on interethnic differences in body fat distribution and phenotype make it reasonable to find different adipokine levels in different ethnic groups, particularly when different cutoff values for waist circumferences have to be considered [17].

It is interesting to note that, in another study involving Caucasian PCOS women from Southern Italy, FF-leptin concentrations were found to be predictive of the fertilization rates [15]. In PCOS women, the fertilization rate is known to be lower than in normally ovulating women, and therefore it may seem reasonable to find reduced FF-leptin levels in these subjects.

In our study, both NOW-PCOS and control-NOW had normal glucose tolerance. Fasting insulin levels and HOMA-IR index were not significantly different in the two groups. However, circulating insulin levels correlated with FF-leptin in NOW-PCOS only, even if in both NOW-PCOS and control-NOW they correlated with BMI.

It has been already demonstrated that hyperleptinemia and/or high FF-leptin may result in impaired steroidogenesis, reproductive function, and fertility [15, 33, 34]. However, it is also true that follicle maturation occurs in the presence of physiologic levels of leptin through the activation of the STAT signal transduction pathway [13, 15]. The possibility exists that too low levels of FF-leptin decrease fertility in NOW-PCOS women as well as too high FF-leptin levels might favor infertility in overweight/obese PCOS women. In overweight PCOS women, insulin resistance is certainly associated with increased steroidogenesis, even if the adipose cell lineage is not intrinsically insulin-resistant [35, 36].

As far as visfatin is concerned, it belongs to the family of cytokines and has been implicated in several metabolic conditions, such as central obesity, metabolic syndrome, and type 2 diabetes. In an animal model (mouse), administration of visfatin during superovulation improves developmental competency of oocytes and fertility potential, despite the age. These and other data support a role for visfatin in reproduction [18, 19]. In our experimental cohort, serum visfatin levels were not different in NOW-PCOS and control-NOW, but FF-visfatin levels were 1.6-fold higher, even though not significantly, in NOW-PCOS as compared with control-NOW.

In regard to the IVF stimulation procedure, even if fertilization rate has been known to be lower in PCOS women as compared with normally ovulating women, due to IVF, the fertilization rate resulted to be not different in the two groups of women analysed in our study. Since during the IVF protocol, NOW-PCOS and control-NOW followed a lifestyle change program including a Mediterranean low-glycemic index isocaloric diet and daily moderate exercise, according to standard care protocols, we can argue that this lifestyle change might have resulted in improved fertilization rates in women with PCOS, even if estrogen levels were still higher in NOW-PCOS than in control-NOW.

TABLE 2: Outcomes at the stimulation end in the experimental women.

	NOW-PCOS	Control-NOW	p value
Follicles <18 mm	8.14 ± 3.07	2.58 ± 2.48	2.11
Follicles >18 mm	6.24 ± 2.02	6.05 ± 2.55	0.8
17β-E ₂ (pg/mL)	3184.26 ± 1477.09	1689.67 ± 1075.79	0.001
Total oocytes	9.33 ± 3.28	5.95 ± 2.86	0.001
M II	3.76 ± 1.37	4.89 ± 2.26	0.07
M II/total oocytes (%)	45.38 ± 22.78	81.23 ± 23.73	2.12
Fertilization rate (%)	60.16 ± 35.22	62.82 ± 33.77	0.81
Embryos (I°)	1.74 ± 1.52	2.05 ± 1.47	0.52
Pregnancy rate (%)	30	35	

NOW: nonoverweight; control-NOW: controls; BMI: body mass index; E₂: estradiol; M II: in-vitro-matured metaphase II oocytes. Data are expressed as mean ± SEM. Differences between groups were considered significant for $p < 0.05$.

A possible take-home message to get from our pilot study is that NOW-PCOS might represent a completely different phenotype as compared with those found in overweight or obese women with PCOS. FF-leptin levels lower and FF-visfatin levels higher than those found in control-NOW might be markers of this different phenotype and low fertilization rate.

Considering the correlation between insulin circulating levels and FF-leptin in NOW-PCOS, future studies should more closely focus on direct measurements of insulin sensitivity and insulin secretion in NOW-PCOS to better elucidate the relationship between changes in insulin secretion and/or action and changes in cytokines relevant to the fertility status in this population.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

G. Garruti, R. de Palo, M. T. Rotelli, S. Nocera, I. Totaro, C. Nardelli, M. A. Panzarino, M. Vacca, L. E. Selvaggi, and F. Giorgino equally contributed to the paper.

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Research Article

Macrophages and Leydig Cells in Testicular Biopsies of Azoospermic Men

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A number of studies have indicated that testicular macrophages play an important role in regulating steroidogenesis of Leydig cells and maintain homeostasis within the testis. The current paper deals with macrophages (CD68 positive cells) and Leydig cells in patients with nonobstructive azoospermia (NOA). Methods employed included histological analysis on semi- and ultrathin sections, immunohistochemistry, morphometry, and hormone analysis in the blood serum. Histological analysis pointed out certain structural changes of macrophages and Leydig cells in NOA group of patients when compared to controls. In the testis interstitium, an increased presence of CD68 positive cells has been noted. Leydig cells in NOA patients displayed a kind of a mosaic picture across the same bioptic sample: both normal and damaged Leydig cells with pronounced vacuolisation and various intensity of expression of testosterone have been observed. Stereological analysis indicated a significant increase in volume density of both CD68 positive and vacuolated Leydig cells and a positive correlation between the volume densities of these cell types. The continuous gonadotropin overstimulation of Leydig cells, together with a negative paracrine action of macrophages, could result in the damage of steroidogenesis and deficit of testosterone *in situ*.

1. Introduction

Within the mammalian testis, there is a significant population of macrophages situated in the interstitial compartment. They have Fc and complement receptors, express macrophage-specific markers, produce interleukins, phagocytose and kill pathogenic organisms. These cells also incorporate trypan blue, plutonium, and demonstrate acid phosphatase and nonspecific esterase activity [1–7]. Morphological studies on the rat testis have revealed a specific ultrastructural coupling of macrophages and Leydig cells. Namely, Leydig cells project slender cytoplasmic processes towards deep coated channels

of macrophages. The channels are limited by an electron dense part of the macrophage membrane. It can be supposed that these channels could be sites of an intensive exchange of molecules/signals between the two cell populations [1, 8, 9]. In the adult normal human testis, macrophages reside in the testis interstitium and are by no means encountered within seminiferous tubules [10]. However, in the biopsies of infertile men, these cells (apart from their interstitial location) can be seen within the lamina propria and seminiferous epithelium and/or lumen of the tubules [10–12]. Leydig cells are the major source of testosterone [13] but also are capable of producing many nonsteroidal factors like β -endorphin [14]

and prodynorphin [15], which are known to act as paracrine factors affecting macrophage function.

Infertility affects in average 15–20% couples and is, therefore, a major health problem. Incidence of male infertility is rising due to various genetic, infectious, and environmental factors. The most difficult patients to treat are those presenting with azoospermia (no spermatozoa in the ejaculate). Approximately 8% of infertile men are diagnosed either with obstructive (OA) or nonobstructive azoospermia (NOA) and are frequently subjected to (micro)surgical spermatozoa retrieval (testicular biopsy) and testicular sperm extraction (TESE). In general, OA is characterized by an intact testicular parenchyma, maintained production of spermatozoa within seminiferous tubules, and normal morphology of the interstitial tissue. Yet, after a longer period of obstruction, some macrophages could be found within the seminiferous epithelium [16, 17]. NOA cases, however, demonstrate various degrees of seminiferous tubules damage: hypospermatogenesis, disturbance of spermatogenic cells maturation at the level of spermatids (spermatid “stop”), or spermatocytes (spermatocyte “stop”) up to drastic pathological changes such as Sertoli cells only syndrome and/or tubular fibrosis [18]. Sometimes, in the histological sections of NOA testicular biopsies, a combination of the above-mentioned pathology can be seen, recognized as a “mixed atrophy” of seminiferous tubules [19]. In the case of NOA, the structure of Leydig cells and the production of testosterone are frequently changed when compared to controls [20–22]. In the biopsies of infertile men, Leydig cells are often hypertrophic and/or hyperplastic and diffusely arranged around hypoplastic seminiferous tubules [23, 24]. The levels of testosterone could be either decreased or increased, depending on the nature of Leydig cells changes within the gonad. If Leydig cells are poorly differentiated from their mesenchymal precursors, they are not capable of maintaining the normal testosterone production. However, in many cases testosterone levels are maintained because Leydig cells are sufficiently differentiated and stimulated by LH. FSH and LH are frequently increased in infertile men with disturbance of spermatogenesis [25, 26]. In addition, the presence of mast cells in such bioptic material proved to be abundant [27]. These cells are found to be increased in various forms of NOA, including mixed atrophy [28–30]. Study of Bergh [31] demonstrated that macrophages and Leydig cells responded to unilateral cryptorchidism in a similar fashion. In the cryptorchid testis, both macrophages and Leydig cells were reduced in size and number.

Recent papers on testicular macrophages and Leydig cells interactions mostly deal with animal or *in vitro* models [32–35]. Since nonobstructive azoospermia is the form of male infertility largely difficult to treat, the aim of our study was to investigate morphological features of macrophages and Leydig cells in testicular biopsies of infertile men that were affected by that particular disorder. Moreover, we wanted to draw a parallel between the expression of testosterone production/expression *in situ* with morphological characteristics of macrophages and Leydig cells, as well as the levels of testosterone and gonadotropins in the blood serum of azoospermic patients.

2. Materials and Methods

2.1. Testicular Biopsy. Overall, 120 patients with azoospermia that consulted andrologist for male infertility in the period from 1998 to 2006 at the Clinic of Urology (University of Zagreb, School of Medicine, University Centre Zagreb) were included into the study. All patients were subjected to an open biopsy of the testis [36, 37]. Prior to the biopsy, patients gave their written and informed consent to surgery and biopsy examination. The current study has been approved by an appropriate ethical committee. A detailed diagnostic procedure preceded the testicular biopsy, including minimum 2–3 semen analyses in order to confirm azoospermia. The testis volume has been determined by ultrasound. Whenever possible, a bilateral biopsy was performed. Briefly, an incision of 8–10 mm in length in the t. albuginea of the testis has been made. This incision allowed 4–5 testicular lobules to be included into the biopsy. The protruding testicular tissue was dissected using surgical microscissors. Typically, 5 pieces were taken from different parts of the male gonad. After dissection, 4 pieces were immediately fixed in Bouin's fluid and 1 piece in a buffered 5.5% glutaraldehyde [18]. Careful histological analysis identified twelve patients with fully preserved spermatogenesis according to the Johnsen's score [25] and normal morphology of the interstitial tissue. Based on their clinical presentation and histology analysis, those patients were diagnosed with obstructive azoospermia (OA) and formed a control group ($N = 12$). The average age of patients was 34 years (range 24–37 years). The rest of the patients ($N = 108$) displayed various degrees of damage of a testicular parenchyma: hypospermatogenesis, spermatid and spermatocyte “stop,” spermatogonia only syndrome, picture of Sertoli cells only syndrome, tubular sclerosis, or combination of the aforementioned testicular disorders known as “mixed atrophy” [19]. Those patients were diagnosed with nonobstructive azoospermia (NOA). The average age of patients was 32 years (range 22–43 years).

2.2. Tissue Processing. Tissue fixed in glutaraldehyde was rinsed several times in 0.05 M phosphate buffer (pH = 7.1–7.4, 800 mOsm) and postfixed with 1% OsO_4 . The tissue was then dehydrated in a series of ascending alcohol concentrations. After a routine histological procedure, the testicular tissue was embedded in Durcupan (Agar). Semithin sections (section thickness = $0.9 \mu\text{m}$) were made by Reichert ultramicrotome and stained with 1% toluidine blue. Sections were covered with a drop of Merckoglass (Merck) and a coverslip. Ultrathin sections were made (Reichert ultramicrotome, section thickness = 70 nm), contrasted with lead citrate and uranyl acetate, and examined by a transmission electron microscope Zeiss 902A (Centre for Electron Microscopy, Medical School University of Zagreb).

Small pieces of testicular tissue (similar to the size of a rice) fixed in Bouin's fluid were dehydrated. For a morphometric (stereological) part of the study, the tissue was allowed to be oriented in a random fashion and embedded in paraffin blocks. Each block was trimmed and oriented (also in a random fashion) for cutting. The blocks were then cut extensively by a rotary microtome (Leitz). Part of serial sections for a

routine bright field microscopy (section thickness = 4 μm) was placed on slides and stained with hemalaun and eosin (H&E). After staining, slides were inspected by a binocular microscope Eclipse 200 (Nikon). The unstained sections were prepared for immunohistochemistry.

2.3. Immunohistochemistry. Unstained paraffin sections were placed on silane-coated slides. Slides were then incubated for 20 minutes at 60°C. Sections were deparaffinized and incubated for 3 \times 5 minutes in 10 mmol/L citrate buffer (pH 6.0) in a microwave oven at 800 W and 400 W powers for antigen retrieval [38]. Subsequently, tissue slides were washed with PBS buffer (pH 7.2) and the endogenous peroxidase activity was blocked by a 5-minute treatment with H_2O_2 . Slides were then washed with PBS buffer and preadsorbed for 15 min with 20% mouse serum. Afterwards, the slides were incubated in a humid chamber for 30 minutes with a primary antibody at a room temperature. Following primary antibodies were used according to the manufacturer's instructions: CD68 (macrophage/monocyte marker, 1:100, Dako, Glostrup, Denmark) and testosterone (1:50, Biogenex, San Ramon, USA). After washing in PBS buffer, the secondary biotinylated antibody (or the appropriate kit with an envision complex) was added for 30-minute incubation. Slides were then washed with PBS buffer and treated with streptavidin-horseradish peroxidase for 30 minutes. Tissue sections were washed once more in PBS buffer and then chromogen 3,3'-diaminobenzidine was added for 5 minutes. Slides were washed in distilled water, stained with hemalaun for 1 minute, washed with water, dehydrated with alcohol (96%), cleared with xylene, and mounted with Entellan (Merck, Darmstadt, Germany). As a negative control, omission of primary antibodies was used.

2.4. Morphometric (Stereological Analysis). For a stereological analysis, a nonbiased Weibel's 42-point multipurpose test system was applied at a magnification of $\times 400$ [39]. The length of the test lines was 0.045 mm, and the test surface area was 0.0736 mm^2 for each analyzed microscopic field. The slides that underwent immunohistochemistry staining were placed under the microscope. They were focused (without refocusing). In order to determine the volume (or volume density, V_v) of immunopositive cells (CD68 positive cells and testosterone producing cells) in a unit volume of testicular tissue, a point counting method has been used. To determine V_v the following formula was applied [40–42]:

$$V_v = \frac{P_c}{P_t}, \quad (1)$$

where P_c is number of hits on positive cells whereas P_t is a number of test points ($P_t = 42$). A pilot stereological measurement of V_v has been made in order to determine the number of microscopic fields (n) needed for a statistically reliable data assessment. The pilot measurement has been carried out on 20 microscopic fields. After this preliminary measurement, de Hoff's formula was applied [40–42]:

$$n = \left(20 \times \frac{s}{x} \right)^2, \quad (2)$$

where x is the mean value of V_v and s is the standard deviation. In our case, the number of microscopic fields to be assessed for each patient was 20–50, depending on the results of the pilot measurement/s.

2.5. FSH, LH, and Testosterone Blood Serum Levels. The levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone in blood serum were determined using standard reagents (kits) provided by Ortho-Clinical Diagnostics (Johnson & Johnson, Amersham, UK). A Vitros ECI device has been used. Reagents contained a set of 100 microwells with antibody for the appropriate hormone/s bound to horseradish peroxidase, antibody to hormone/s bound to biotin, calibrators, signal reagent, and washing solution. An enhanced chemiluminescence method has been applied.

2.6. Statistical Analysis. Data were statistically analysed using Statistica 10.0 software developed by StatSoft. The statistically significant difference between the control and NOA group of patients was determined using nonpaired Student's t -test. $P < 0.05$ indicated a statistically significant difference between the two groups. Based on the aforementioned test and the results of general statistics package, graphs and a table representing mean values and standard errors of means were generated. In addition, Pearson's correlation coefficient between volume density of CD68 positive cells (V_v , CD68+) and vacuolated Leydig cells (V_v , Lc) was determined along with statistical significance of correlation.

3. Results

3.1. Qualitative Histological Analysis on Semithin Sections. Control biopsies from patients with OA displayed regular morphology of the testicular parenchyma: seminiferous tubules had regular diameter (200–220 μm), normal thickness of the lamina propria (10–15 μm), and basement membrane and well-developed seminiferous epithelium. The epithelium consisted of supportive Sertoli cells and all kinds of spermatogenic cells: spermatogonia, spermatocytes, round and elongated spermatids, and spermatozoa (Figure 1(a)). The loose connective tissue of the interstitial compartment predominantly consisted of fibrocytes and fibroblasts. Within this tissue, clusters of Leydig cells were noted. Leydig cells had conspicuous round or oval nucleus with sometimes prominent nucleolus. Their cytoplasm was abundant, bearing few lipid droplets and sometimes one or two vacuoles. Small blood vessels like arterioles, venules, and capillaries were found interwoven in the Leydig cell clusters, in the close proximity of seminiferous tubules, or in the wall of the lamina propria (which was especially true for capillaries) (Figure 1(a)). Depending on their location, Leydig cells could be subdivided into peritubular (closer to seminiferous tubules) or perivascular (closer to blood vessels in the middle of the interstitial space). Large cells residing within the interstitium, close to Leydig cells, with a kidney-shaped or

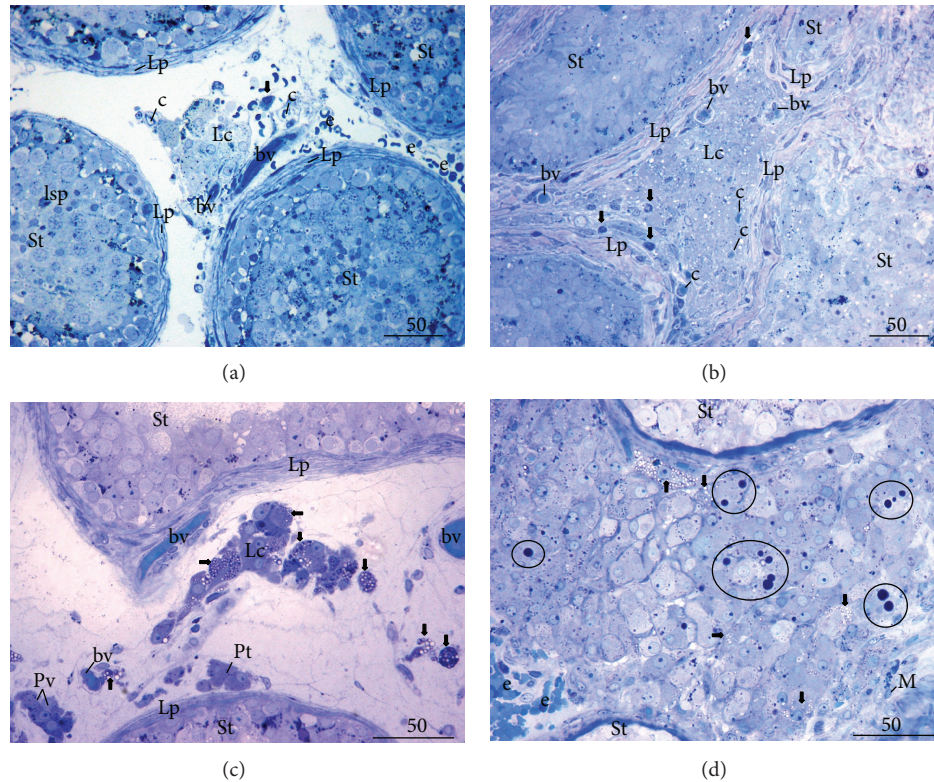


FIGURE 1: Semithin sections of the control testicular biopsy (obstructive azoospermia case, OA) (a) and biopsies from patients with nonobstructive azoospermia (NOA) ((b)–(d)). (a) Between several seminiferous tubules (St) with regular spermatogenesis, a part of the testicular interstitium with a cluster of Leydig cells (Lc) is visible. Leydig cells have a regular, round nucleus surrounded by an abundant cytoplasm. In the close proximity of these cells, a presumptive macrophage (↓) and several small blood vessels (bv) including capillaries (c) are noted (Lp, lamina propria; lsp, late spermatid; e, extravasated erythrocytes). (b) A part of testicular biopsy from a patient with NOA. Seminiferous tubules (St) are lined with Sertoli cells only and have a thickened lamina propria (Lp). Leydig cells (Lc) in the middle demonstrate a high vacuolisation of the cytoplasm. Presumptive macrophages (↓) are arranged at the periphery of seminiferous tubules or between Leydig cells. Several small blood vessels (bv) as well as capillaries (c) are also found. (c) Testicular biopsy from a patient with a spermatocyte “stop.” According to their location/related to seminiferous tubules (St) and blood vessels (bv)/, Leydig cells can be subdivided into peritubular (Pt) and perivascular (Pv). These cells demonstrate regular morphology, in contrast to many vacuolated Leydig cells (→) in the center (Lc) (Lp, lamina propria). (d) Testicular biopsy (NOA patient, spermatocyte “stop”) with Leydig cell hypertrophy/hyperplasia. Within the abundant population of Leydig cells, those with vacuolated cytoplasm (→) and large lipid droplets (encircled areas) can be observed (St, seminiferous tubules; M, presumptive macrophage; e, extravasated erythrocytes) (Toluidine blue, $\times 400$ /(a), (b)/, $\times 600$ /(c), (d)/, scale bar = 50 μm).

indented nucleus and well-developed cytoplasm were classified as “presumptive macrophages.” Frequently, within the interstitial space, extravasated erythrocytes were observed as a consequence of a surgical procedure (Figure 1(a)).

NOA group of patients was represented by various histological pictures: within some biopsies, small number of mature or elongated spermatids together with abundant presence of round spermatids and other spermatogenic cells were seen (spermatid “stop”); some biopsies were depleted of spermatids and spermatogenesis went up to the spermatocyte stage (spermatocyte “stop”). In some cases, only spermatogonia and Sertoli cells were found within the seminiferous epithelium (spermatogonia only) or the epithelium consisted only of Sertoli cells (“Sertoli cells only” syndrome). Small proportion of NOA patients (3%) had tubular sclerosis where all seminiferous tubules were transformed into the fibrous tissue or “tubular shadows.” However, in the vast majority of

the cases (68% of NOA patients) a combination of the above-mentioned histological pictures could be recorded, which is known as a “mixed atrophy” of seminiferous tubules.

NOA patients also displayed a variety of histological changes within the interstitial tissue (which accompanied various degrees of the damage of seminiferous tubules). Frequently, the loose connective tissue of the interstitial compartment was densely packed with fibrocytes, macrophages, and Leydig cells together with small blood vessels (Figure 1(b)). In general, NOA patients had a kind of mosaic Leydig cells picture across the same bioptic sample: in some areas Leydig cells had a regular appearance; in other areas, their morphology has been changed (Figures 1(b)–1(d)). The interstitial tissue sometimes bore smaller Leydig cells in size (when compared to the control group). Within the cytoplasm of these cells, numerous small translucent vacuoles were identified (Figure 1(b)). In other areas (i.e., of the same

biopsy) Leydig cells were of mixed morphology: some preserved their typical round or oval nucleus with rich cytoplasm and few small lipid droplets. Others, however, had plentiful vacuoles and small or large lipid droplets (Figures 1(b)–1(d)). Sometimes, a focal or diffuse Leydig cells hypertrophy was observed. Seminiferous tubules were surrounded by many large Leydig cells. In the cytoplasm of these hypertrophic cells, numerous vacuoles and/or large lipid droplets were identified. The vacuoles were either located in one part of or diffusely spread throughout the cell cytoplasm (Figure 1(d)). In semithin sections, presumptive macrophages were found in the vicinity of the lamina propria of seminiferous tubules (or within it) or intermingled with Leydig cells (Figure 1(b)). Sometimes, their cytoplasm was loaded with phagocytized material (Figure 1(d)).

3.2. Transmission Electron Microscopy (TEM). In control biopsies, testicular macrophages (that were occasionally present within clusters of Leydig cells or in their proximity) had an indented nucleus with plenty of euchromatin. Few patches of heterochromatin were found within the euchromatin or typically associated with the nuclear membrane. A prominent Golgi apparatus, a lot of mitochondria with cristae, cisternae of predominantly rough endoplasmic reticulum, and few lipid droplets were identified within the cytoplasm. In addition, macrophages in control biopsies had moderate number of primary and secondary lysosomes and occasional residual/lipofuscin bodies. The borders between macrophages and nearby Leydig cells were clearly defined by their cell membranes. No cellular extensions of Leydig cells towards macrophages were recorded (Figures 2(a) and 2(b)).

When compared to macrophages in control biopsies, the same type of cell in NOA group bore much more secondary lysosomes, residual and lipofuscin bodies, and cellular extensions (Figure 2(c)). Within secondary lysosomes and lipofuscin bodies, a variety of phagocytized material has been observed (Figure 2(d)).

Leydig cells of the control group were recognized by a large, round nucleus and frequently prominent nucleolus. The nucleus was regular (mostly without indentations), rich with euchromatin whereas heterochromatin was arranged along the nuclear membrane. Well-developed cytoplasm contained a lot of organelles. Cells were surrounded by an extracellular matrix rich in collagen fibres (Figure 3(a)). Inside the cytoplasm, a lot of cisternae of smooth endoplasmic reticulum were seen. Small numbers of cisternae of rough endoplasmic reticulum were also observed. Leydig cells were rich in mitochondria with tubular cristae. Few lipid droplets and one or two vacuoles were recorded as well (Figure 3(b)).

The “mosaic picture” of Leydig cells in NOA biopsies was also confirmed by TEM. Significant number of cells was normal in appearance, with typical ultrastructure as the cells in control biopsies. In contrast to those cells, some Leydig cells in the group of patients with NOA had a nucleus with pronounced indentations. The euchromatin had more patches of heterochromatin and nucleolus was frequently absent (Figure 3(c)). Furthermore, changed cells had much more lipid droplets varying in size and a lot of vacuoles. Vacuoles seemed to have a unit membrane and content with

a low electron density, apart from the thin area along the rim of the vacuole. That area frequently displayed material of a moderate electron density. The majority of cisternae of smooth endoplasmic reticulum were wider than normal, although the same cell could have areas of the cytoplasm with normal cisternae as well. The morphology of mitochondria was found to be preserved (Figures 3(c) and 3(d)).

3.3. Results of Immunohistochemistry (IHC) Analysis. Control slides with preadsorption and omission of primary antibodies (either for CD68 or testosterone) proved to be negative. No positive cells were found in seminiferous tubules and testicular interstitial compartment (Figure 4(a)). Within control testicular biopsies, CD68 was expressed in a moderate number of cells within the loose connective tissue of the interstitium. CD68 positive cells were surrounded by other connective tissue cells. Few of such cells were found in the vicinity of the lamina propria of seminiferous tubules and clusters of Leydig cells (Figure 4(b)). No CD68 positive cell was found within the seminiferous tubules whatsoever (Figure 4(b)).

In the biopsies of NOA patients, CD68 positive cells were abundant in the interstitial tissue. They infiltrated areas with Leydig cells as well as the lamina propria of some seminiferous tubules. Moreover, these cells were found crossing the tubular wall and invading the seminiferous epithelium as well as the tubular lumen. CD68 positive cells in the proximity of blood vessels were also recorded (Figures 4(c) and 4(d)).

In control biopsies, testosterone positive cells were arranged either in larger or smaller groups in the interstitial compartment (Figure 5(a)). Both peritubular and perivascular Leydig cells were found to be positive for testosterone. Although varying in intensity, the signal was homogeneously distributed throughout the whole cluster/s of cells (Figures 5(a) and 5(b)). Leydig cells of NOA biopsies displayed a kind of mixed expression of testosterone. Some of the cells had normal intensity of the signal, whereas some demonstrated a weak and irregular expression of testosterone (Figures 5(c) and 5(d)). Moreover, in some cells the signal was rather strong with a kind of an overexpression of testosterone (Figure 5(d)). The irregular and inhomogeneous signal was especially present in samples where (in semithin sections) many Leydig cells had vacuoles in their cytoplasm.

3.4. Results of Quantitative Analysis: Testis Volume, Status of Spermatogenesis, Stereology Data, and Hormones Levels. Testis volume in patients with nonobstructive azoospermia was significantly decreased in comparison to the control group ($P < 0.001$) (Table 1). The same goes for the status of spermatogenesis measured by Johnsen's score. In NOA group of patients, the spermatogenesis was significantly impaired when compared to controls ($P < 0.001$) (Table 1).

Volume density determined for CD68 positive cells showed statistically significant difference between the control and NOA group of patients. In NOA group, this stereological variable had significantly higher value than in controls ($P < 0.001$) (Figure 6). NOA group also displayed a significantly higher volume of vacuolated Leydig cells when compared to the control group ($P < 0.05$) (Figure 7). The correlation analysis pointed out a significant positive correlation between

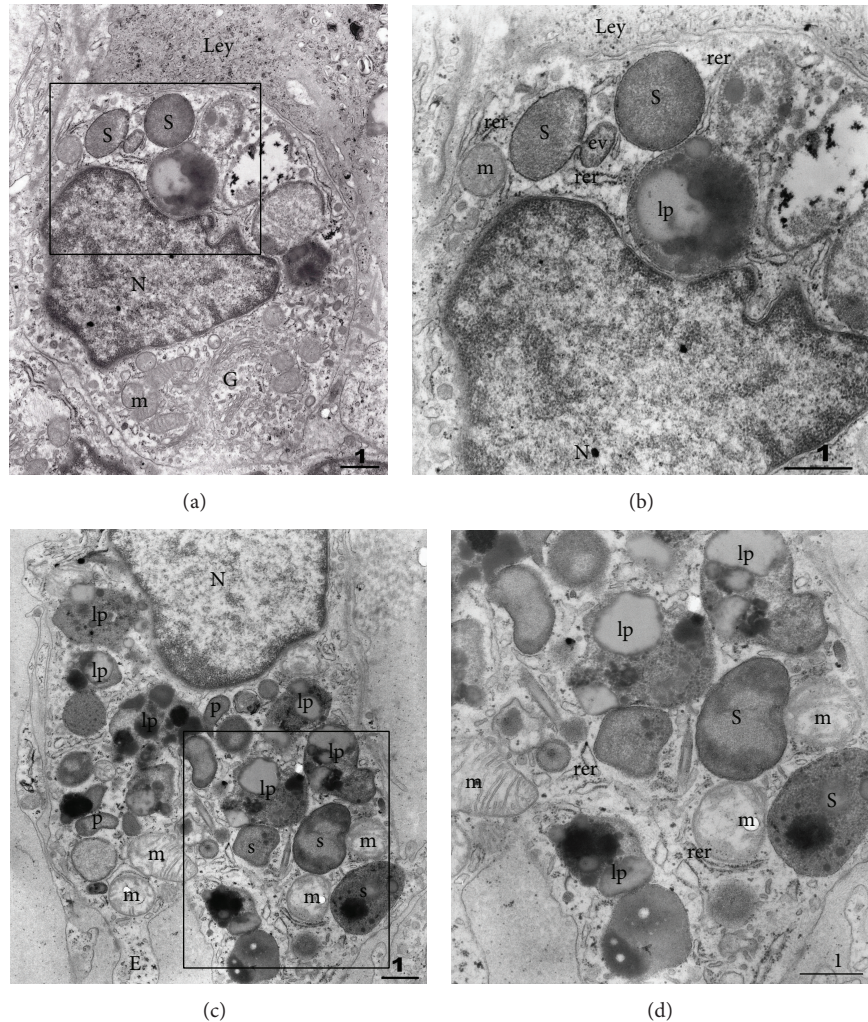


FIGURE 2: Transmission electron microscopy (TEM) of testicular macrophages in the control biopsy ((a), (b)) and in the case of NOA ((c) and (d)). (a) Macrophage with indented nucleus (N) is facing the neighbouring Leydig cell (Ley). Perinuclear region bears a well-developed Golgi apparatus (G). Several mitochondria (m) are found in the vicinity of the apparatus. Within another part of the cytoplasm, secondary lysosomes (s) are visible. The framed area is shown in (b). (b) The cytoplasm of the macrophage opposing Leydig cell (Ley) is rich in secondary lysosomes (s) and lipofuscin bodies (lp). Endocytic vacuole (ev) is in a process of a fusion with one secondary lysosome (N, nucleus; m, mitochondrion; rer, rough endoplasmic reticulum). (c) A macrophage from NOA case. A part of the nucleus (N) is visible, surrounded by an organelle-rich cytoplasm that bears a lot of primary (p) and secondary (s) lysosomes. Numerous lipofuscin bodies and phagosomes (lp) demonstrate intensive phagocytic activity of the cell (m, mitochondria; E, cellular extension; framed area represented in (d)). (d) Between secondary lysosomes (s) and lipofuscin bodies (lp) some scattered cisternae of rough endoplasmic reticulum (rer) and mitochondria (m) could be noted (TEM, original magnification $\times 7,000$ /(a), (c)/, $\times 12,000$ /(b), (d)/; scale bar = $1 \mu\text{m}$).

the volume density of CD68 positive cells and the volume density of Leydig cells with vacuoles ($r = 0.852434$, $P < 0.001$) (Figure 8). In contrast to the results described above, stereological analysis showed no statistically significant difference in the volume density of cells positive to testosterone between control and NOA group of patients ($P > 0.05$), although the volume density of cells expressing testosterone *in situ* was higher in the control group of biopsies (Figure 9).

The analysis of blood serum levels of gonadotropins (FSH, LH) demonstrated significantly higher levels of these hormones in NOA group of patients ($P < 0.01$, $P < 0.001$, resp.). Control group of patients had normal levels of gonadotropins (Table 1). Testosterone serum levels were

within the normal range in the control group of patients. The vast majority of NOA patients had normal levels of testosterone as well. However, in a number of NOA patients ($N = 11$), testosterone levels lower than normal range have been noted. In contrast, small group of patients ($N = 3$) had increased levels of testosterone. There was no statistically significant difference between control and NOA group in testosterone levels ($P > 0.05$) (Table 1).

4. Discussion

There is a growing body of evidence that testicular macrophages play an important role in regulating steroidogenesis

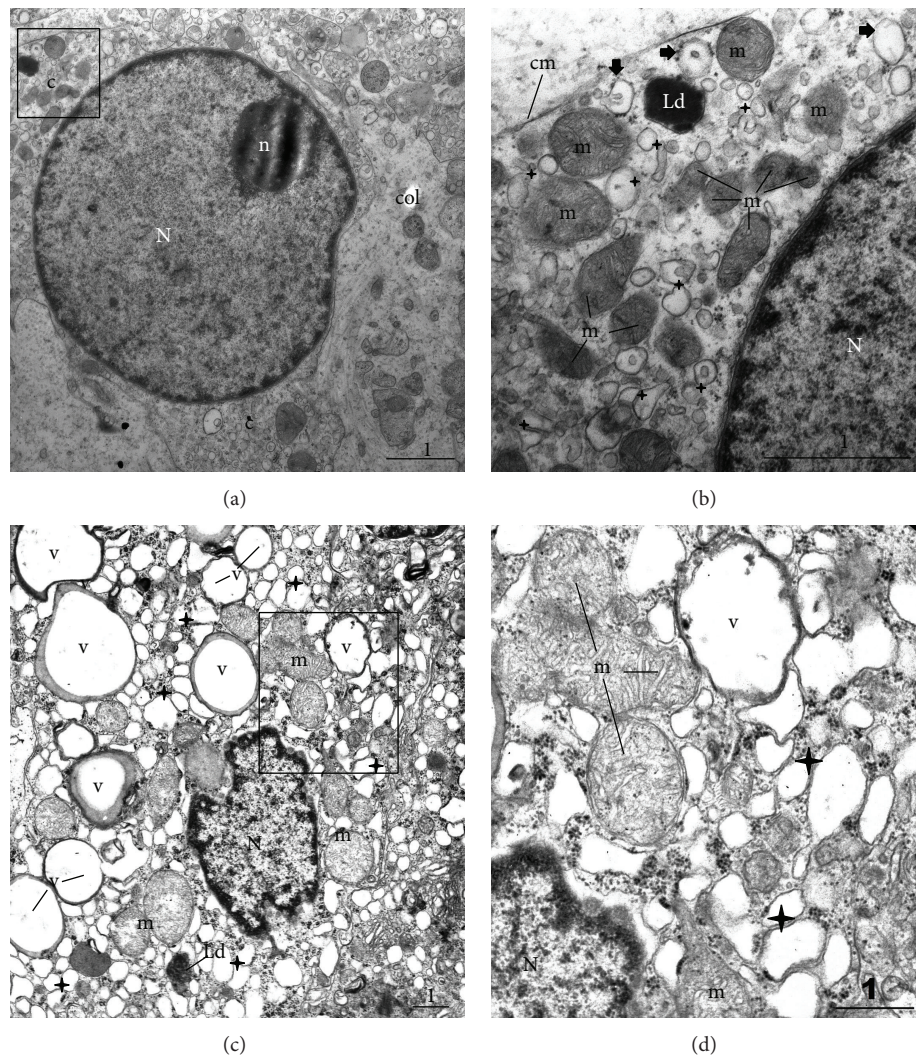


FIGURE 3: Transmission electron microscopy of a Leydig cell in the control biopsy ((a), (b)) and in the case of NOA ((c) and (d)). (a) Leydig cell from the control biopsy. Within a regular, rounded nucleus (N) a prominent nucleolus (n) is found. Cytoplasm (c) contains various organelles. The cell is surrounded by an extracellular matrix with collagen fibres (col). Framed area is represented in (b). (b) Part of the Leydig cell depicted in the previous picture. In the close proximity of the nucleus (N), numerous mitochondria (m) with tubular cristae, many cisternae of smooth endoplasmic reticulum (♦), and a lipid droplet (Ld) are visible. Occasional polyribosomes (→) are bound to few cisternae of rough endoplasmic reticulum (cm, cell membrane). (c) Leydig cell from the biopsy of NOA patient. The nucleus (N) is heavily indented and irregular. The cytoplasm bears a lot of vacuoles (v) with a low electron density material. Other typical organelles for a steroid-producing cell are also present: mitochondria (m), cisternae of smooth endoplasmic reticulum (♦), and sporadic lipid droplets (Ld). The framed area is represented in (d). (d) A part of Leydig cell shown in the previous figure, higher magnification. In the vicinity of the nucleus (N), a cell cytoplasm has a vacuole (v) with a kind of a unit membrane. The majority of the vacuole is translucent. However, the rim contains a thin area of the material of moderate electron density (also present in other vacuoles illustrated in (c)). Some of the cisternae of smooth endoplasmic reticulum (♦) are wider than normal (m, mitochondria) (TEM, original magnification $\times 7.000$ /(a), (c)/, $\times 20.000$ /(b), (d)/; scale bar = $1\mu\text{m}$).

of Leydig cells and maintain homeostasis within the testis [43–45]. Under normal physiological and noninflammatory conditions, macrophages play an important role in Leydig cell development. If macrophages are absent from the testicular interstitium, Leydig cells do not succeed in developing normally, which suggests that macrophages provide important growth and differentiation factors for Leydig cells. In infertile men with damage of spermatogenesis and/or chronic orchitis, when macrophages are activated and secrete an array

of inflammatory mediators, Leydig cell steroidogenesis is inhibited [43].

Despite a significant progress in the area of reproductive medicine and high curing rates, NOA remains to be a considerable problem. As pointed by our results as well, NOA is often characterized by marked pathological changes in both tubular and interstitial compartment. NOA patients could suffer from a testosterone deficit. If the levels of testosterone drop below 15 nmol/L , a change in mood, depression, and a

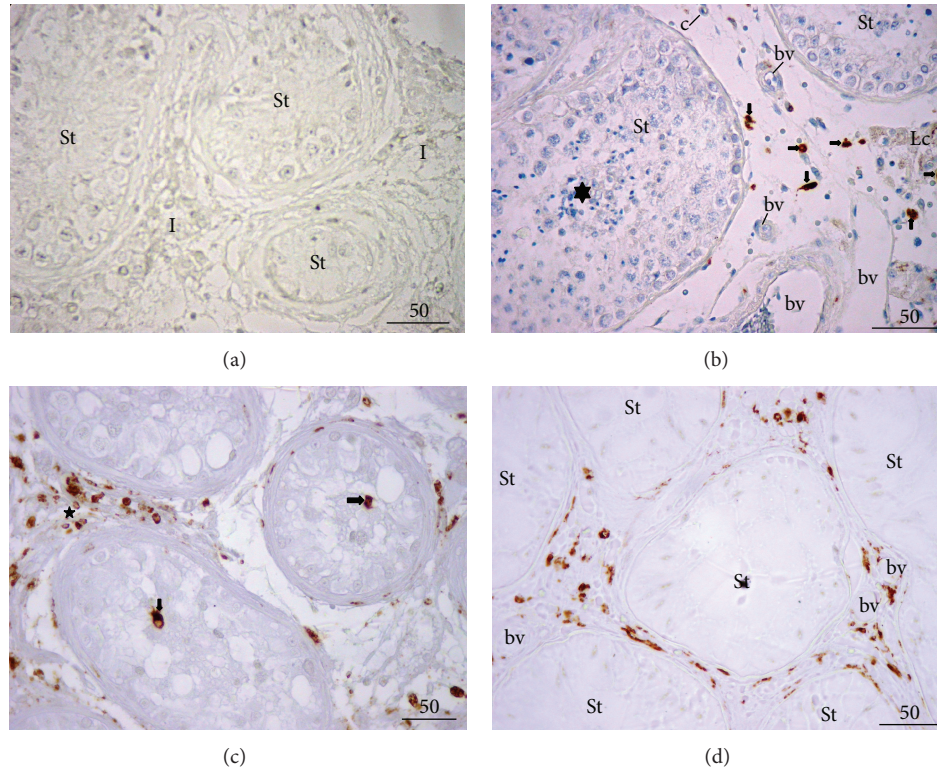


FIGURE 4: Expression of a monocyte/macrophage marker, CD68. (a) Control of immunohistochemistry (IHC) reaction, preadsorption, and omission of a primary antibody. No signal is present either in the testicular interstitium (I) or seminiferous tubules (St). (b) Control biopsy with full spermatogenesis. Within the seminiferous tubules (St), a lot of elongated spermatids (★) are visible. Between seminiferous tubules, a moderate presence of macrophages (CD68 positive cells, →) is notable. None of these cells are found within seminiferous tubules (Lc, cluster of Leydig cells; bv, blood vessels). (c) A biopsy from a patient with NOA. Seminiferous tubules (lined with Sertoli cells only or occasional spermatogonia) are surrounded by a loose connective tissue heavily loaded with CD68 positive cells (★). Some of these cells are found attached to the lamina propria, or invading the lumen of the tubules (→). (d) NOA patient. Seminiferous tubules (St) show a picture of Sertoli cells only syndrome. The testis interstitium is rich with CD68 positive cells (3,3'-diaminobenzidine (DAB), hemalaun counterstain, $\times 400$, scale bar = $50\ \mu\text{m}$).

loss of libido have been noted. The levels of testosterone below $8\ \text{nmol/L}$ cause erectile dysfunction. Other symptoms include adiposity, insomnia, concentration deficit, diabetes mellitus type 2, and, in the long run, osteoporosis [46, 47]. Thus, it is important to have an insight into the macrophage-Leydig cells interaction in NOA patients. The current study aims to provide data on morphological changes of Leydig cells (and decreased production of testosterone *in situ*) in the presence of increased macrophage population (CD68 positive cells) in NOA patients, which could also clarify the above-mentioned clinical observations.

In our study, the vast majority of NOA patients had a significantly decreased testis volume. This was in a way supported by data assessed on semithin sections, where a pronounced loss of various spermatogenic cells has been recorded. When the status of spermatogenesis has been assessed by Johnsen's score, this score was significantly lower in NOA patients. Semithin sections have also provided an insight into the morphology of interstitial tissue (that is frequently neglected in the routine work because of the search for spermatozoa in seminiferous tubules). In semithin sections of testicular biopsies of NOA patients, a pronounced

vacuolisation of some Leydig cells and a notable presence of macrophages loaded with phagocytized material have been recorded. Macrophages were present not only in the close proximity of seminiferous tubules, but they were also increasingly "intermingled" with Leydig cells. The above-mentioned observations were strongly supported by our immunohistochemistry and morphometric data. Thus, in NOA group, multitudinous CD68 positive cells were found in the interstitial compartment, infiltrating the lamina propria of seminiferous tubules as well as seminiferous epithelium. Since CD68 antibody identifies monocyte/macrophage lineage, the observed CD68 positive cells are very likely recruited from blood and infiltrated interstitial and tubular testis compartments, in addition to resident macrophages that were already present in the loose connective tissue of the testis.

Namely, it seems that the population of testicular macrophages, at least in the rat, is not a homogenous one. The majority of testicular macrophages exhibit a cell-surface antigen, which is recognised by monoclonal antibody ED2. This antigen is expressed exclusively on fixed tissue-resident macrophages [48, 49]. However, a small population of testicular macrophages do not have detectable ED2 antigen.

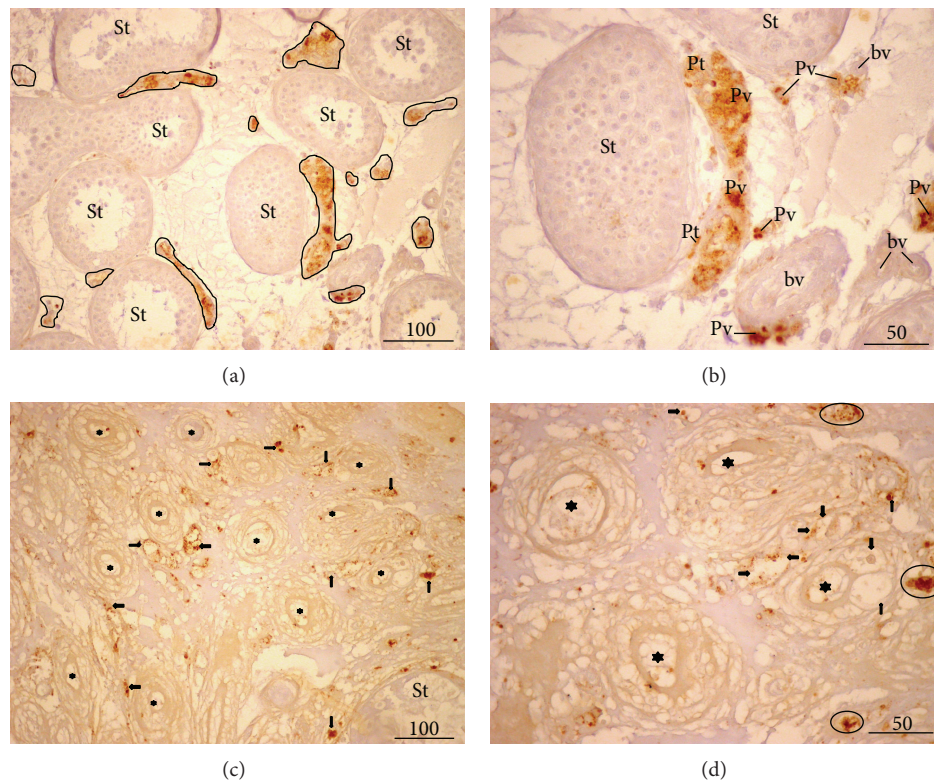


FIGURE 5: Testosterone expression *in situ*. (a) Control biopsy. Larger or smaller groups of Leydig cells are positive to testosterone (encircled areas) (St, seminiferous tubules). (b) Testosterone expression, details of the previous figure. A strong signal is present both in peritubular (Pt) as well as perivascular Leydig cells (Pv) (St, seminiferous tubules; bv, blood vessels). (c) NOA, testicular biopsy with tubular sclerosis (★) and intensive vacuolisation of Leydig cells. An irregular expression of testosterone (→) is notable (St, seminiferous tubule). (d) NOA, higher magnification. Some Leydig cells demonstrate highly scattered and irregular positive signal (→); in contrast, few of them possess normal or very strong signal (encircled cells) (★, fibrosed tubules, the so-called “tubular shadows”) (3,3'-diaminobenzidine (DAB), hemalaun counterstain, $\times 200$, scale bar = $100\ \mu\text{m}$ /(a), (c); $\times 400$, scale bar = $50\ \mu\text{m}$ /(b), (d)).

These macrophages can be identified because they are positive for the antigen recognised by antibody ED1 (CD68), a marker of both circulating monocytes and macrophages. Approximately half of the ED2 positive resident testicular macrophages lack detectable expression of ED1. It is very likely that this marker is lost after the cells become resident in the testis [48, 49]. Freshly isolated testicular macrophages from normal rats produce relatively low levels of the inflammatory cytokines interleukin-1 beta and tumour necrosis factor- α in response to various stimuli [50–52]. These data suggest that testicular macrophages, at least in the rat, are poorly proinflammatory. In humans, interleukin-1 alpha has been related with orchitis, relapse of acute lymphoblastic leukemia in the testis, and infertility disorders in men [53].

The results of our study, in particular its immunohistochemistry and morphometry part, concur to those presented by Frungieri et al. [11], where an increased number of CD68 positive cells were clearly identified in the loose connective tissue of the interstitium and in the lamina propria of hypoplastic seminiferous tubules as well as within the seminiferous epithelium and tubular lumen. Based on these findings, it has been postulated that testicular macrophages both directly (via phagocytosis) or indirectly (via paracrine

modulators) have been involved in the regulation of steroidogenesis. Our study clearly pointed out some changes in the Leydig cell fine structure in the increased presence of CD68 positive cells. Stereological analysis emphasized that there was an increased presence of CD68 positive cells and vacuolated Leydig cells in NOA group of patients. Moreover, a positive correlation between these two cell groups has been found: the more the CD68 positive cells, the more the vacuolated Leydig cells.

Our results, based on the histological analysis of NOA Leydig cells in semithin sections, pointed out that these cells underwent both hypertrophy and hyperplasia. Apart from Leydig cells with large, abundant cytoplasm loaded with lipid droplets and/or vacuoles, there were also some smaller forms. These cells were elongated, much resembled to fibroblasts but expressed testosterone and, ultrastructurally, had cell organelles of steroid-producing cells. Frequently, across the same NOA biopsy, both such “types” of Leydig cells could be found. In the morphometric study of Tash et al. [24], it has been claimed that Leydig cells in NOA patients undergo hypertrophy only and their number remains constant when compared to OA cases. The measurements have been based on morphometric data and decreased testis volume of NOA

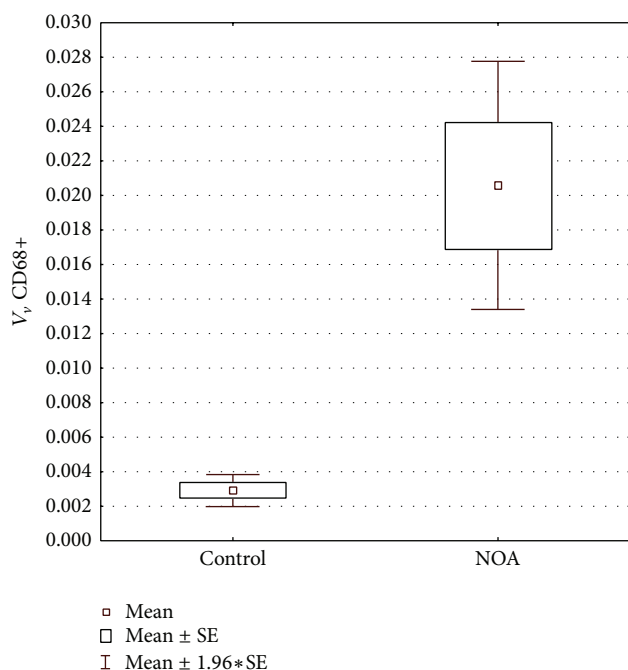


FIGURE 6: Volume density of CD68 positive cells ($V_v \text{ CD68+}$) in control and NOA group of patients. Volume density of these cells in NOA group is significantly increased ($P < 0.001$).

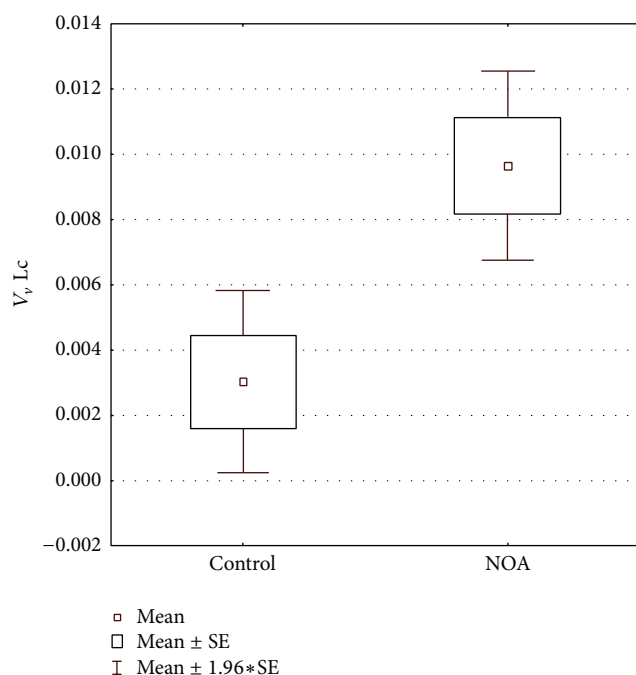


FIGURE 7: Volume density of vacuolated Leydig cells in control and NOA group. Volume is significantly increased in the group of patients with nonobstructive azoospermia ($P < 0.05$).

patients, which was also presented in our study. However, the decrease of the testis volume is mainly due to spermatogenic cell loss and tubular hypoplasia, and, to the lesser extent, due to changes within the testis interstitium. The small “type” of Leydig cells that we noticed in semithin sections could be

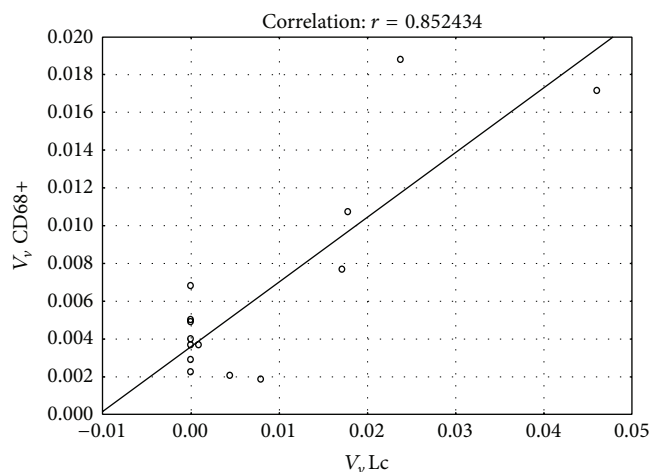


FIGURE 8: Correlation between volume density of CD68 positive cells and volume density of vacuolated Leydig cells. A statistically significant positive correlation has been found ($r = 0.852434$, $P < 0.001$).

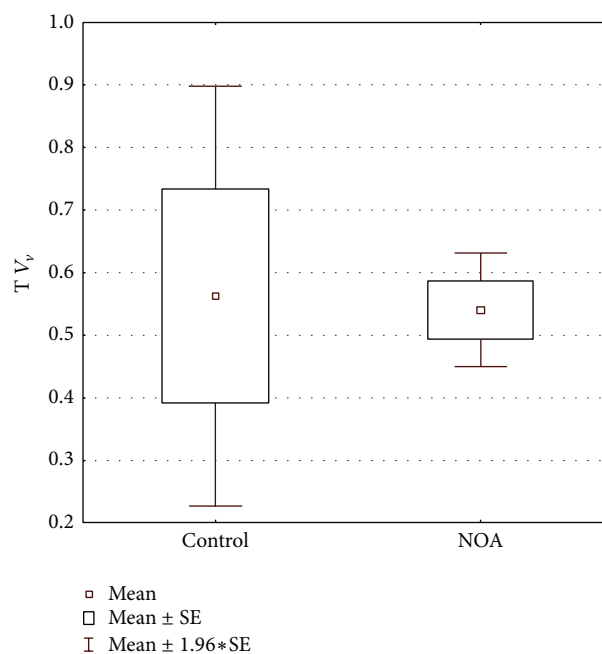


FIGURE 9: Volume density of testosterone immunopositive cells in control and NOA group. There is no statistical difference between the investigated groups ($P > 0.05$).

developed from mesenchymal precursors that are present in the loose connective tissue of the interstitial compartment. Thus, the question of Leydig cell hypertrophy/hyperplasia should be further explored.

A number of studies also pointed out the association between macrophages and mast cells and possible involvement of both cell types in the fibrotic changes of seminiferous tubules [11, 28–30]. In one of our previous papers [27] we presented similar observations, particularly in relation to NOA cases with “mixed atrophy” of seminiferous tubules. Although there are data on the interactions between

TABLE 1: Testis volume, score of spermatogenesis, and hormone values in the control group (OA) and in patients with nonobstructive azoospermia (NOA).

	OA Vo (cm ³)	NOA Vo (cm ³)	OA score	NOA score	OA FSH (UI/L)	NOA FSH (UI/L)	OA LH (UI/L)	NOA LH (UI/L)	OA T (nmol/L)	NOA T (nmol/L)
\bar{x}	19.737	8.123	9.582	4.131	8.55	19.74	5.04	10.06	11.46	12.49
s	1.866	3.707	0.163	2.052	1.628	1.58	3.10	4.12	3.838	5.89
SE	0.311	0.618	0.027	0.342	3.51	2.59	0.93	0.92	1.16	1.32
Max	22.43	16.34	8.857	8.171	10.60	51.9	10.00	18	18.60	271
Min	16.34	4.03	8.314	1	2.23	5	1.60	4.97	5.20	2.6
P (OA/NOA)	<0.001		<0.001		<0.01		<0.001		>0.05	

\bar{x} : mean; s: standard deviation; SE: standard error of mean; Max: maximal value; Min: minimal value; P (OA/NOA): statistical significance between OA and NOA.

macrophages and mast cells [11], the exact influence of mast cells on Leydig cells and steroidogenesis remains to be further investigated.

Our transmission electron microscopy analysis has demonstrated that macrophages of NOA patients contain a significant number of secondary lysosomes, residual and lipofuscin bodies, and cellular extensions. The ultrastructural analysis demonstrated the existence of large vacuoles within the cytoplasm of Leydig cells. The vacuoles were translucent with low electron density. They seemed to have a unit membrane and were accompanied by wide cisternae of smooth endoplasmic reticulum. The nature of vacuoles and their exact content remain to be further investigated. The vacuole could be a product of a fusion of several wide cisternae of smooth endoplasmic reticulum. At the rim of the vacuoles, along the unit membrane, a material of a moderate electron density has been frequently observed. It could be speculated that the above described vacuoles contain lipids or inhomogeneous material with lipids component/s. It should be mentioned at this point that, at the ultrastructural level, typical lipid droplets do not have a unit membrane. Moreover, vacuoles have been recorded either in one part of Leydig cell or they were diffusely arranged throughout the entire cytoplasm. Thus, normal cell machinery (especially smooth endoplasmic reticulum and mitochondria where important steps of steroidogenesis take place) is replaced by huge vacuoles. It can be speculated that the highly vacuolated Leydig cells with wide cisternae of smooth endoplasmic reticulum are only partially effective in terms of testosterone production. A negative influence of nearby located activated macrophages could add to testosterone deficiency *in situ*. This has been supported by our immunohistochemistry data (expression of testosterone). In NOA patients, some Leydig cells had a normal pattern of testosterone expression, similar to those in controls. In other cells (observed as highly vacuolated in semithin sections) the signal was largely irregular and decreased. One can speculate that vacuoles, which occupy a significant part of the cytoplasm, are negative for testosterone and cause the above-mentioned irregular pattern of expression or no expression at all. On the other hand, in some Leydig cells an overexpression of testosterone has been found.

The analysis of hormone levels in blood serum indicated high levels of gonadotropins (FSH, LH) in NOA patients. This concurs to the results of our previous study [25] and others dealing with NOA [54–56]. Thus, Leydig cells are continuously stimulated by LH. One can assume that the constant “bombardment” with gonadotropins, together with paracrine factors of macrophages, causes damage of Leydig cells. Overstimulation of these cells could provoke building of wide cisternae of smooth endoplasmic reticulum and huge vacuoles described in the current paper. All these events can create a kind of “burn-out” syndrome for Leydig cells. The deficit of testosterone *in situ* could act negatively on spermatogenesis, causing loss of spermatogenic cells and infertility. The results of our immunohistochemistry and morphometric analysis as well as testosterone levels in the blood support such a scenario. The measurement of the volume density of testosterone producing cells indicated no significant difference between control and NOA group,

although the control group had slightly higher values of immunopositive Leydig cells. This lack of significant difference could be due to the (hyper)stimulation of LH. Thus, NOA patients have, in the vast majority of cases, normal values of testosterone. Overstimulated Leydig cells as well as those cells that preserved their normal structure and function (seen in semithin sections) could produce enough testosterone to maintain the normal serum levels. In addition, overstimulated Leydig cells could produce an excess of testosterone, which explains a small proportion of NOA patients that had higher levels of testosterone and overexpression of this hormone recorded in the immunohistochemistry part of our study. Finally, few NOA patients would have “exhausted,” burn-out Leydig cells and, therefore, lower levels of testosterone in blood than normal. Thus, in NOA patients, CD68 positive cells act negatively of Leydig cells. What is the exact trigger of such a pattern in NOA remains to be elucidated.

5. Conclusion

The current paper deals with macrophages (CD68 positive cells) and Leydig cells in patients with nonobstructive azoospermia (NOA). Histological analysis pointed out certain structural changes of macrophages and Leydig cells in NOA group of patients when compared to controls. These changes included an increased presence of CD68 positive cells. Leydig cells in NOA patients displayed a kind of a mosaic picture: both normal and damaged Leydig cells with pronounced vacuolisation and various intensity of expression of testosterone have been observed across the same bioptic samples. There was a positive correlation between the volume density of CD68 positive and vacuolated Leydig cells. It has been speculated that the continuous gonadotropin stimulation of Leydig cells, together with the negative paracrine action of macrophages on these testosterone producing cells, could result in the damage of steroidogenesis and deficit of testosterone *in situ*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

New Tools for Embryo Selection: Comprehensive Chromosome Screening by Array Comparative Genomic Hybridization

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The objective of this study was to evaluate the usefulness of comprehensive chromosome screening (CCS) using array comparative genomic hybridization (aCGH). The study included 1420 CCS cycles for recurrent miscarriage ($n = 203$); repetitive implantation failure ($n = 188$); severe male factor ($n = 116$); previous trisomic pregnancy ($n = 33$); and advanced maternal age ($n = 880$). CCS was performed in cycles with fresh oocytes and embryos ($n = 774$); mixed cycles with fresh and vitrified oocytes ($n = 320$); mixed cycles with fresh and vitrified day-2 embryos ($n = 235$); and mixed cycles with fresh and vitrified day-3 embryos ($n = 91$). Day-3 embryo biopsy was performed and analyzed by aCGH followed by day-5 embryo transfer. Consistent implantation (range: 40.5–54.2%) and pregnancy rates per transfer (range: 46.0–62.9%) were obtained for all the indications and independently of the origin of the oocytes or embryos. However, a lower delivery rate per cycle was achieved in women aged over 40 years (18.1%) due to the higher percentage of aneuploid embryos (85.3%) and lower number of cycles with at least one euploid embryo available per transfer (40.3%). We concluded that aneuploidy is one of the major factors which affect embryo implantation.

1. Introduction

Aneuploidies are common in early human embryos [1, 2]. Trisomic and monosomic embryos account for at least 10% of human pregnancies and, for women nearing the end of their reproductive lifespan, the incidence may exceed 50% [3]. Further, aneuploidy rates are higher in oocytes and embryos from women with advanced maternal age (AMA) [4] which probably stems from meiotic recombination defects exacerbated by age [5]. Recent studies in humans and model organisms have shed new light on the complexity of meiotic defects, providing evidence that the age-related increase in errors in human females is not attributable to a single factor but to an interplay between the unique features of oogenesis

and a host of other endogenous and exogenous factors [3]. Age-related defects result in higher aneuploidy rates in offspring and an increase in spontaneous abortions, thereby reducing ongoing implantation rates [6]. Aneuploidy may also be a contributing factor in other infertile populations; for example, despite other potential causes, an abnormal embryonic karyotype was found to be the most frequent cause of recurrent miscarriage (RM) [7]. In the same study, the percentage of cases with RM of truly unexplained origin was limited to 24.5%. While the diagnosis of repetitive implantation failure (RIF) remains a clinical challenge (its causes can be multiple, often with ill-defined embryonic and endometrial contributing factors), embryonic aneuploidy has been proposed as one of the leading embryonic

causes [8]. In male factor (MF) infertility, an increase in sperm chromosomal abnormalities due to impairment of the meiotic process has been described [9, 10]. Additionally, a higher incidence of abnormal karyotypes has been described in the miscarriages of couples undergoing intracytoplasmic sperm injection (ICSI) because of MF infertility [11].

Despite a meta-analysis compiling nine randomized controlled clinical trials (RCTs) [12] indicating that there is no benefit to preimplantation genetic screening (PGS) by fluorescence *in situ* hybridization (FISH) for a limited number of chromosomes, some controversial opinions have surfaced regarding the convenience for embryo aneuploidy screening [13–19], including our own experience which differs from previously published studies. We conducted two prospective, randomized trials to evaluate the usefulness of PGS in AMA patients between 41 and 44 years of age and RIF patients aged less than 40 years of age. We observed a significant increase in the live birth rates in the PGS group compared to the blastocyst group in the AMA study (32.3% versus 15.5%; $P = 0.0099$) and a clear trend towards increased live birth rates in the RIF study (47.9% versus 27.9%). We therefore concluded that PGS with classic FISH is beneficial for these two indications if proper blastomere biopsy procedures and good laboratory conditions are applied [20].

Despite our findings, there is still a clear need for a technique capable of comprehensive chromosome screening (CCS), which could also produce reliable and faithful results in a short period of time. The first approach was comparative genomic hybridization (CGH), and several studies were published using this technology [21–23]. However, in the last three years, embryo aneuploidy screening has evolved: it is more broadly applied in *in vitro* fertilization (IVF) programs and now includes other approaches that allow results to be obtained in a shorter period of time, such as oligoarrays, single nucleotide polymorphism arrays, quantitative PCR, and CGH bacterial artificial chromosome arrays (SNP, qPCR, and CGH BAC, resp.) [24–33]. In two recently published reviews, array-CGH (aCGH) was described as a robust and accessible diagnostic approach to assess 24-chromosome aneuploidy, and hence IVF programs are moving towards PGS using aCGH [34, 35].

Independent of the type of platform used, the technique selected for screening all 24 chromosomes should offer reliable and timely results and should only be applied in clinical programs after validation with an already well-established technique. In our program, we first validated the aCGH platform in single cells from embryos previously diagnosed as abnormal by FISH, obtaining similar error rates below three percent for both techniques [36]. Next, we confirmed that there were no differences in efficiency and accuracy when comparing day-3 and day-5 whole embryo array analysis [30]. This was further validated in another study using the same aCGH platform confirming the high efficiency of the platform: only 2.9% of embryos had no results, and the error rate when compared to FISH was 1.9% [37]. In the work presented here, we describe our current experience with the clinical application of CCS using aCGH for different clinical indications, considering oocyte and embryo vitrification as a

coadjuvant technique to improve reproductive outcomes in IVF patients.

2. Materials and Methods

2.1. Patients. This retrospective study compiled 1420 cycles with a day-3 biopsy in which aCGH analysis was performed, from February 2010 to February 2013. Clinical indications for CCS were the following: RM (two or more miscarriages of unknown etiology); RIF (three or more previous IVF failures); MF (low sperm concentration or a significant increase in sperm chromosomal abnormalities); couples with a previous trisomic pregnancy (PTP); and AMA (40 years or older). The study included different cycle types: cycles in which all oocytes and embryos came from fresh cycles ($n = 774$); mixed cycles with fresh and vitrified oocytes ($n = 320$); mixed cycles with fresh and vitrified day-2 embryos ($n = 235$); and mixed cycles with fresh and vitrified day-3 embryos ($n = 91$). The goal of vitrification at different stages was to increase the number of embryos for the analysis within a single CCS cycle.

2.2. Embryo Biopsy and Culture Conditions. Patients underwent ovarian stimulation using standardized protocols. When at least two follicles reached 18 mm in diameter, recombinant human chorionic gonadotropin (hCG, Ovitrelle, 250 mg, Merck Serono, Geneva, Switzerland) was administered, and oocyte retrieval was scheduled 36 hours later. ICSI was performed in all cases [38]. Fertilization was assessed 17–20 hours after microinjection, and embryo cleavage was recorded every 24 hours. The CCS cycles were performed in different IVF centers using two main protocols. In most centers, embryos were grown in IVF/CCM medium (1/1) (Vitrolife, Göteborg, Sweden) until day 3 and were subsequently cultured in CCM medium with a monolayer of endometrial epithelial cells until day 5 [39]. In the remaining centers, global sequential culture system (LifeGlobal, Guilford, CT) was used with tri-gas incubators (7% O_2 and 5% CO_2).

Embryo biopsy was performed on day 3 and can be summarized as follows: embryos were placed on a droplet containing Ca^{2+}/Mg^{2+} -free medium (G-PGD, Vitrolife, Göteborg, Sweden/LifeGlobal, Guilford, CT), the *zona pellucida* was perforated using laser technology (OCTAX, Herborn, Germany), and one blastomere was withdrawn from each embryo. Only embryos with five or more nucleated blastomeres and less than 25% fragmentation were biopsied. Individual blastomeres were placed in 0.2 mL PCR tubes containing 2 μ L PBS. For blastomere washing and handling, 1% polyvinylpyrrolidone (PVP) was used. Properly developed euploid embryos were transferred on day 5, and surplus euploid embryos were vitrified either on day 5 or on day 6.

2.3. Oocyte and Embryo Vitrification. The Cryotop method was used as previously described by Kuwayama et al. (2005) [40] and adapted for our laboratory [41]. In brief, oocytes/embryos were immersed in a solution containing

7.5% (v/v) ethylene glycol (EG) with 7.5% (v/v) dimethylsulfoxide (DMSO) in TCM199 medium with 20% (v/v) synthetic serum substitute (SSS) at room temperature for 15 minutes. Subsequently, oocytes/embryos were placed in a solution containing 15% EG with 15% DMSO and 0.5 mol/L sucrose. One minute later, they were placed on the Cryotop strip and immediately submerged in filtered liquid nitrogen (Brymill filter model 9409, Brymill Corporation, Ellington, CT, USA). For warming, the Cryotop was removed from the liquid nitrogen and instantly placed in 1.0 M sucrose in TCM199 with 20% SSS at 37°C. After 1 minute, oocytes/embryos were placed in 0.5 mol/L sucrose in TCM199 with 20% SSS at room temperature for 3 minutes. Finally, two consecutive washes (5 minutes and 1 minute) were performed with TCM199 with 20% SSS at room temperature before oocytes were incubated at 37°C for two hours preceding ICSI.

2.4. DNA Amplification and Array-Comparative Genomic Hybridization Protocol. To perform day-3 aCGH analysis, a single cell from each embryo was amplified using the Sureplex DNA amplification system (BlueGnome, Cambridge, UK). Amplification quality was ensured by gel electrophoresis (Lonza, Rockland, USA). Sample and control DNA were labelled with Cy3 and Cy5 fluorophores following the manufacturer's instructions. Labelling mixes were combined and hybridized on 24sure arrays (V2 and V3, BlueGnome, Cambridge, UK) for 6–12 hours. Each probe is specific to a different chromosomal region and occupies a discrete spot on the slide. Chromosomal loss or gain is revealed by the color adopted by each spot after hybridization. The technique involves the competitive hybridization of differentially labeled test and reference DNA samples. Fluorescence intensity was detected using a laser scanner (Powerscanner, TECAN, Männedorf, Switzerland), and BlueFuse Multi software was used for data processing (BlueGnome, Cambridge, UK). The "24sure microarray product description (February 8, 2012, document version 2.3, and model number 408501-00)" describes 10 Mb effective resolution for 24sure using BlueFuse software, being this the minimum size specified by BlueGnome for segmental aneuploidies. The entire protocol can be completed in less than 24 hours and, therefore, embryo transfer and vitrification of surplus euploid embryos can be scheduled for day 5.

2.5. Statistics. The chi-square test and Fisher exact test were used for comparisons between study groups with respect to percentages. Welch *t*-test was used to compare noncategorical variables. Bonferroni's correction for multiple group comparisons was applied and $P < 0.05$ was considered statistically significant. The implantation rate was defined as the percentage of embryos transferred resulting in an implanted gestational sac. The pregnancy rate per transfer was calculated as the percentage of clinical pregnancies with a fetal heart beat. The miscarriage rate was defined as the percentage of clinical pregnancies that were spontaneously miscarried before week 12 of pregnancy. The delivery rate per cycle was defined as the number of cycles with a live birth.

3. Results and Discussion

3.1. General Clinical Results. A total of 1420 CCS cycles were performed. The mean female age was 39.4 (SD 3.4). A total of 7210 embryos were analyzed and, in 7118 (98.7%) of them, amplification and further analysis were successful. A high percentage of aneuploid embryos were observed for all 24 chromosomes (77.6%), with 3.9% of them showing segmental aneuploidies defined as gains or losses of chromosome fragments with size larger than 10 Mb. A chaotic pattern was observed in 15.0% of the embryos. In 783 cycles, at least one euploid embryo was available for transfer, with a pregnancy rate of 53.4% per transfer and 29.4% per cycle. The miscarriage rate was 7.4%, and the delivery rate per cycle was 27.3%. The results from our CCS program using aCGH technology support our previous experience of the benefits of using FISH for a limited number of chromosomes. In fact, the average pregnancy rate per transfer for all indications was higher than in our previously published studies using FISH for similar indications, which produced pregnancy rates ranging between 30 and 40% [42–44]. Therefore, the incorporation of aCGH in our aneuploidy screening program has resulted in a clear increase in pregnancy and implantation rates, showing that aneuploidies for any of the 24 chromosomes can appear in preimplantation embryos and therefore can impair embryo viability and implantation.

3.2. Clinical Results according to the Origin of Oocytes and Embryos. Table 1 summarizes our results according to the origin of oocytes and embryos. Comparisons among the four groups showed a similar mean female age. The mean number of MII oocytes was 9.0 (SD 4.9) in the group of fresh oocytes; 10.1 (SD 4.7) in the vitrified oocytes group; 12.4 (SD 6.6) in the group of day-2 vitrified oocytes, and 12.8 (SD 6.9) in the day-3 vitrified embryo group. The informative and aneuploid embryos as well as their clinical outcomes were similar among groups, showing that vitrification had no detrimental clinical impact at any stage compared to fresh cycles. Statistical differences were only observed in the mean number of embryos analyzed (which was significantly higher for vitrified oocyte and vitrified day-2 embryo groups compared to fresh and day-3 vitrified cycle groups) and in the percentage of cycles reaching the embryo transfer stage, which was lower for vitrified oocytes compared to day-2 vitrified cycle groups.

A high proportion of cycles (62%) were performed in women who were aged 40 years or more. For this reason, in women with low ovarian response, the goal of vitrification was to accumulate a sufficient number of MII oocytes or embryos to be able to achieve embryo transfer and subsequent ongoing pregnancy. Oocyte vitrification from different stimulation cycles for oocyte accumulation has been successfully applied to low-responder patients in regular IVF cycles [45] and the introduction of vitrification in IVF programs opens new possibilities for embryo selection and CCS [46, 47]. Additionally, another recent publication showed that the process of oocyte vitrification does not increase embryonic aneuploidy and does not impact implantation [48]. An alternative for achieving an optimal number of embryos for

TABLE 1: Clinical results of 1420 CCS cycles according to the origin of the oocytes and embryos.

	Fresh cycles	Mixed vitrified oocytes	Mixed day-2 vitrified embryos	Mixed day-3 vitrified embryos
Number of cycles	774	320	235	91
Mean female age (SD)	39.4 (3.2)	39.7 (3.9)	38.9 (3.5)	38.3 (3.4)
Mean number of embryos analyzed (SD)	4.7 (2.9) ^{b,c}	5.0 (2.7) ^{a,d,c}	6.4 (3.4) ^{a,b,d}	4.4 (2.7) ^{b,c}
Number of informative embryos	3573	1565	1499	481
Total number of aneuploid embryos (%)	2757 (77.2)	1248 (79.7)	1151 (76.8)	371 (77.1)
Number of segmental aneuploidies (%)	151 (4.2)	51 (3.2)	56 (3.7)	20 (4.1)
Number of embryos with a chaotic pattern (%)	561 (15.7)	250 (16.0)	204 (13.6)	53 (11.0)
Number of embryos with >1 aneuploidy (%)	1191 (33.3)	578 (36.9) ^d	485 (32.3)	144 (29.9) ^b
Number of embryo transfers (%)	421 (54.4)	156 (48.7) ^c	147 (62.5) ^b	59 (64.8)
Mean transferred embryos (SD)	1.4 (0.5)	1.4 (0.5)	1.5 (0.5)	1.5 (0.5)
Number of pregnancies/transfer (%)	225 (53.4)	93 (59.6)	71 (48.3)	29 (49.1)
Number of pregnancies/cycle (%)	225 (29.1)	93 (29.1)	71 (30.2)	29 (31.9)
Implantation rate	48.1	51.9	40.0	43.7
Miscarriage rate	6.6 (15/225)	8.6 (8/93)	8.4 (6/71)	6.9 (2/29)
Delivery rate/cycle	271 (210)	26.6 (85)	27.7 (65)	29.7 (27)

^a $P < 0.05$ versus fresh cycles; ^b $P < 0.05$ versus mixed vitrified oocytes; ^c $P < 0.05$ versus mixed day-2 vitrified embryos; ^d $P < 0.05$ versus mixed day-3 vitrified embryos. Chi-square test, Fisher exact test, or Welch t -test with Bonferroni's correction.

biopsy is vitrification at the cleavage stage, which has also shown optimal performance in a long retrospective study [49]. However, a recent study of aCGH cycles showed that the cohort size was not significantly associated with the euploidy rate [50]. In this present study, the group of mixed vitrified oocytes showed the highest percentage of embryos with more than one aneuploidy (36.9%), which may be due to the older age of the women included in this group. Embryo vitrification at any of the other stages before the biopsy did not have an impact on the percentage and distribution of different types of chromosomal abnormalities. Similar aneuploidy rates were obtained in fresh cycles (77.2%) compared to mixed cycles with vitrified oocytes (79.7%), day-2 (76.8%), or day-3 (77.1%) cleavage-stage embryos; no differences were observed in pregnancy, implantation, or delivery rates. These data are comparable to those recently published regarding cycles with fresh and vitrified oocytes from our ovum donation program. Similar metabolomic profiles were also observed in embryos derived from fresh and vitrified oocytes, supporting the feasibility of accumulating oocytes or embryos for a single CCS cycle analysis [51].

3.3. Clinical Results according to the Comprehensive Chromosome Screening Indication. Table 2 shows the results for the different infertility indications studied. The mean number of embryos analyzed was significantly higher for the MF indication compared to the other indications, with a lower number of embryos analyzed for the AMA group (mean 4.6). The percentage of aneuploid embryos was similar for all indications below 40 years of age, with a significant increase in the AMA group (85.3%) compared to all the other groups (68.2% in RM, 67.7% in RIF, 71.5% in PTP, and 65.4% in MF). These results had an impact in the percentage of cycles with at least one euploid embryo for transfer, making it significantly lower for the AMA group (40.3%) compared to all the other indications (77.3% in RM, 79.2% in RIF, 78.8% in PTP, and 83.6% in MF). However, once embryo transfer was achieved, the chances of successful pregnancy and implantation were similar for all the mentioned indications, with a range between 46.0% and 62.9% for pregnancy rates per transfer and between 40.5% and 54.2% for implantation rates. However, the pregnancy rate per CCS cycle was significantly lower for the AMA group (19.3%) compared to the remaining indications (44.3%, 45.2%, 36.4%, and 52.1% for RM, RIF, PTP, and MF, resp.) due to the previously mentioned high transfer-cancellation rate. The delivery rate per cycle was also significantly lower for the AMA group (18.1%) compared to the other indications (38.4% in RM, 43.1% in RIF, 30.3% in PTP, and 50.9% in MF).

Despite the minimal effect of maternal age on implantation after the transfer of a euploid embryo, a negative effect on delivery rates has been described by other authors. A retrospective case-controlled study including CCS cycles with aCGH for PGS in AMA, RM, and RIF patients reported lower ongoing pregnancy rates per cycle in patients 35 years or older compared to patients less than 35 years. However, even in cycles in patients 38 years or older, the implantation, clinical pregnancy, and ongoing pregnancy rates significantly

increased after CCS in these groups compared to their controls [52]. Another multicenter retrospective study described an increase in the incidence of aneuploid embryos, which correlated with increased maternal age, observing similar implantation and ongoing pregnancy rates per transfer after CCS in patients up to 42 years of age, after which these rates dramatically declined [53].

Interestingly, Table 2 shows a different distribution of chromosomal abnormality types according to the indication. The highest incidence of segmental aneuploidies was observed in couples with a previous chromosomally abnormal pregnancy (10.2%) and the lowest was in the AMA group (3.2%). The distribution of embryos showing a chaotic pattern was relatively homogenous among indications, with a slight decrease in RIF patients (11.2%) compared to RM (16.4%) and AMA (15.6%) patients. The most remarkable difference was observed for the percentage of embryos with aneuploidy for more than one chromosome, which was significantly higher in the AMA group (43.1%) compared to all other indications (range: 19.8%–23.5%). This percentage increases with maternal age, reaching values from 32.8% in 40 years to 65.8% in 46 years of age. Therefore, the overall incidence of aneuploidy ranges from 79.0% to 95.7% (Figure 1). This relationship between maternal age and the complexity of aneuploid errors has recently been described by Franasiek et al. (2013) [54] in a systematic report of 15169 CCS results, showing that 36% of embryos had more than one aneuploidy and that the proportion of more complex aneuploidy increases with age.

In RM couples, the transfer of euploid embryos after CCS results in a low miscarriage rate (13.3%). A multicenter study of 287 cycles in couples with idiopathic RM described 60% of the embryos as aneuploid but with a miscarriage rate of 6.9% after CCS, compared to the expected rate of 33.5% in RM control population and 23.7% in an infertile control population [28]. These results showed a clear benefit of 24-chromosome screening in couples with this etiology.

In RIF couples, previous RCTs using FISH for a limited number of chromosomes showed controversial results, with one study showing no clear benefit [55] and another showing an improvement in live birth rates compared to blastocyst transfer without previous FISH analysis [20]. Despite this, there is no RCT regarding CCS with aCGH for RIF patients, although the results described in our study support the application of aneuploidy screening for this group of patients.

In PTP couples, published data describe an increased risk of recurrent aneuploid conceptions, particularly in women under 37 years of age [56]. Previous studies on PGS with FISH analysis of a 9-chromosome panel showed high rates of abnormal embryos, ranging from 48.1% to 71.2% [57, 58], which is in agreement with the percentage of 71.5% observed in our study with 24-chromosome analysis.

Finally, we found the best clinical results after CCS in MF couples. Although, to our knowledge, there are no publications regarding CCS in MF infertile couples, this type of 24-chromosome CCS seems to be a very promising indication for this patient group, as also suggested by previous similar publications with PGS using FISH analysis [42, 59].

TABLE 2: Clinical results of 1420 CCS cycles according to the different indications.

	RM <40 yrs	RIF <40 yrs	PTP <40 yrs	MF <40 yrs	AMA ≥40 yrs
Number of cycles	203	188	33	116	880
Mean female age (SD)	35.9 (2.7)	36.5 (2.5)	36.8 (2.4)	34.8 (3.2)	41.5 (2.1)
Mean number of embryos analyzed (SD)	5.5 (3.1) ^{b,d,e}	5.9 (3.0) ^{a,d,e}	5.7 (3.8) ^{d,e}	6.8 (3.7) ^{ab,c,e}	4.6 (2.6) ^{a,b,c,d}
Number of informative embryos	1099	1064	186	797	3972
Total number of aneuploid embryos (%)	750 (68.2) ^e	720 (67.7) ^e	133 (71.5) ^e	521 (65.4) ^e	3403 (85.3) ^{a,b,c,d}
Number of segmental aneuploidies (%)	61 (5.5) ^e	40 (3.7) ^c	19 (10.2) ^{b,d,e}	32 (4.0) ^c	126 (3.2) ^{a,c}
Number of embryos with a chaotic pattern (%)	180 (16.4) ^b	119 (11.2) ^{a,e}	24 (12.9)	125 (15.5)	620 (15.6) ^b
Number of embryos with >1 aneuploidy (%)	237 (21.6) ^e	250 (23.5) ^e	41 (22.0) ^e	158 (19.8) ^e	1711 (43.1) ^{a,b,c,d}
Number of embryo transfers (%)	157 (77.3) ^e	149 (79.2) ^e	26 (78.8) ^e	97 (83.6) ^e	354 (40.3) ^{a,b,c,d}
Mean transferred embryos (SD)	1.5 (0.5)	1.5 (0.6)	1.4 (0.5)	1.5 (0.5)	1.3 (0.5)
Number of pregnancies/transfer (%)	90 (57.3)	85 (57.0)	12 (46.0)	61 (62.9)	170 (48.0)
Number of pregnancies/cycle (%)	90 (44.3) ^e	85 (45.2) ^e	12 (36.4)	61 (52.1) ^e	170 (19.3) ^{a,b,d}
Implantation rate	479	50.9	40.5	54.2	42.4
Miscarriage rate	13.3	4.7	16.7	3.3	6.5
Delivery rate/cycle	38.4 (78) ^e	43.1 (81) ^e	30.3 (10)	50.9 (59) ^e	18.1 (159) ^{a,b,d}

RM: recurrent miscarriage; RIF: repetitive implantation failure; MF: severe male factor; PTP: previous trisomic pregnancy; AMA: advanced maternal age.

^a $P < 0.05$ versus RM <40 yrs; ^b $P < 0.05$ versus RIF <40 yrs; ^c $P < 0.05$ versus PTP <40 yrs; ^d $P < 0.05$ versus MF <40 yrs; ^e $P < 0.05$ versus AMA ≥40 yrs. Chi-square test, Fisher exact test, or Welch t-test with Bonferroni's correction.

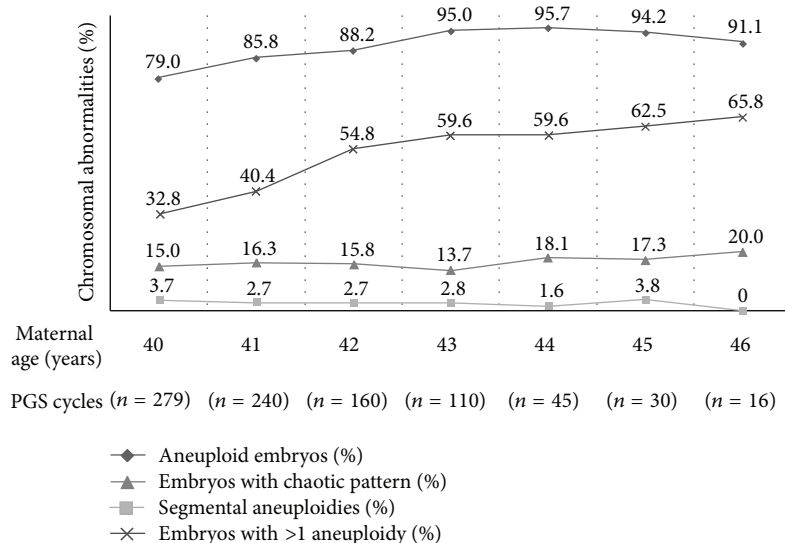


FIGURE 1: Aneuploidy rates according to maternal age in AMA group.

4. Conclusions

Our findings on day-3 embryo biopsies support the basis for CCS in patients in whom a high proportion of aneuploid embryos are suspected. New RCTs should be conducted in the near future to assess the feasibility of using different platforms for different clinical indications and to test for any potential increases in live birth rates resulting from more comprehensive aneuploidy screening before embryo transfer.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Calcium Influx and Male Fertility in the Context of the Sperm Proteome: An Update

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Freshly ejaculated spermatozoa are incapable or poorly capable of fertilizing an oocyte. The fertilization aptness of spermatozoa depends on the appropriate and time-dependent acquisition of hyperactivation, chemotaxis, capacitation, and the acrosome reaction, where calcium (Ca^{2+}) is extensively involved in almost every step. A literature review showed that several ion channel proteins are likely responsible for regulation of the Ca^{2+} uptake in spermatozoa. Therefore, manipulation of the functions of channel proteins is closely related to Ca^{2+} influx, ultimately affecting male fertility. Recently, it has been shown that, together with different physiological stimuli, protein-protein interaction also modifies the Ca^{2+} influx mechanism in spermatozoa. Modern proteomic analyses have identified several sperm proteins, and, therefore, these findings might provide further insight into understanding the Ca^{2+} influx, protein functions, and regulation of fertility. The objective of this review was to synthesize the published findings on the Ca^{2+} influx mechanism in mammalian spermatozoa and its implications for the regulation of male fertility in the context of sperm proteins. Finally, Pathway Studio (9.0) was used to catalog the sperm proteins that regulate the Ca^{2+} influx signaling by using the information available from the PubMed database following a MedScan Reader (5.0) search.

1. Introduction

Spermatozoa are atypical cells with peculiar functionality: they are produced in one organism and released, and then they invade another organism and deliver their genetic material into a host cell to produce offspring by sexual reproduction. It is a well-known fact that only about 1 in 25,000 spermatozoa finally reaches the fallopian tube and gets the opportunity to fertilize an oocyte. In the mid-20th century, it had been claimed that mammalian spermatozoa are unable to fertilize an oocyte before achieving functional maturation, which occurs during their journey through the female reproductive tract for a finite period of time [1, 2]. This fundamental maturational process is chiefly regulated by numerous signaling cascades, and calcium (Ca^{2+}) plays a dynamic role in this process, as an intracellular second messenger. Several studies have hypothesized that elevation of sperm intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$)/ Ca^{2+} influx regulates motility, hyperactivation, chemotaxis, capacitation, and the

acrosome reaction and facilitates the spermatozoa reaching and fertilizing of an oocyte [3–8]. Therefore, understanding the mechanism that regulates the Ca^{2+} influx in spermatozoa is a matter of utmost importance.

Previous studies have shown that the Ca^{2+} entry mechanisms are regulated via numerous Ca^{2+} permeable channel proteins in spermatozoa [6, 9, 10]. Therefore, the factors that regulate the functions of those channels will ultimately help us understand how male fertility is regulated. Recent applications of proteomic approaches such as two-dimensional polyacrylamide gel electrophoresis, mass spectrometry, and differential in-gel electrophoresis have yielded the identification of several sperm-specific proteins [11, 12]. These discoveries have provided new insight into protein functions and enabled us to recognize diverse sperm-specific processes in order to differentiate normal from abnormal spermatozoa [11]. Mature spermatozoa are widely known to be silent in both transcription and translation [11, 13, 14] or poorly capable of translation [15]; therefore, studies on

individual sperm proteomes have described the importance of spermatozoal posttranslational modifications and their ability to induce physiological changes as a prerequisite for successful fertilization.

Torres-Flores et al. [16] have shown that human spermatozoa exposed to the phosphodiesterase inhibitor papaverine cause activation of protein kinase A (PKA) and stimulate the progesterone-induced Ca^{2+} influx via the cyclic adenosine monophosphate- (cAMP-) dependent pathway. Although these authors did not evaluate the relationship between *in vitro* fertility and Ca^{2+} influx, changes in intracellular pH and increased tyrosine phosphorylation ultimately provide a potential clue regarding sperm fertility competence. In another study, to evaluate hamster spermatozoa capacitation capability, comparative association was observed between pyruvate dehydrogenase A, Ca^{2+} influx, cAMP, and reactive oxygen species [17]. Additionally, Breitbart et al. [18] reported that polymerization of globular- (G-) actin to filamentous- (F-) actin occurs during capacitation. As capacitation and the acrosome reaction are Ca^{2+} -mediated events [4, 5], one can, without considering further signaling cascade, assume that remodeling the actin structure might be linked with the regulation of Ca^{2+} influx in spermatozoa.

Recently, in our laboratory, we found that the manipulation of sperm proteins such as ubiquinol-cytochrome-c reductase core protein 2 (UQCRC2) [39], voltage-dependent anion channels proteins (VDACs) [4], and arginine vasopressin [5] could control the Ca^{2+} influx in spermatozoa and regulate capacitation, the acrosome reaction, and fertility. Therefore, design and construction of a similar study with most of the identified sperm proteins available from several protein databases might provide a more realistic insight into the Ca^{2+} influx, protein functions, and fertility. The present work reviews the latest information published by other laboratories as well as our research team on the aforementioned aspects of spermatozoa and their potential implications for diagnosis and prognosis of male fertility.

2. Mechanism of Ca^{2+} Influx in Mammalian Spermatozoa

The ultimate goal of fertilization of mammalian sperm is to fuse with and deliver their genetic materials into an oocyte [2, 40, 41]. For fertilization to occur completely, the spermatozoa must experience various obstacles both *in vitro* and *in vivo* [40, 41]. Ca^{2+} ions act as central signaling molecules; once they enter the spermatozoa, they exert allosteric regulatory effects on enzymes and many proteins [10, 21, 42]. Indeed, numerous elegant research findings have contributed significantly to our understanding of the molecular signaling of Ca^{2+} influx, especially through monitoring the activity of individual cells. However, most of the studies are discrete and often do not represent a cumulative idea. This section presents a compilation of some basic information regarding the Ca^{2+} entry mechanism into mammalian spermatozoa by recapitulating scientific evidence.

The literature reviewed shows that the primary source of Ca^{2+} for spermatozoa is the external environment: the fallopian tube in the female reproductive tract (*in vivo*) and culture media (*in vitro*) [8], and simultaneously increasing $[\text{Ca}^{2+}]_i$ regulates the release of Ca^{2+} into the cell. Therefore, how Ca^{2+} crosses into cells through the sperm plasma membrane is a matter of paramount importance. In eukaryotic cells, the Ca^{2+} influx occurs through specific Ca^{2+} permeable ion channel proteins located on the plasma membrane [43, 44] such as classical voltage-gated (high and low) Ca^{2+} (Ca_v s), transient receptor potential (TRP), and cyclic nucleotide-gated (CNG) channels [9]. Recently, Ren and Xia have proposed four criteria to identify sperm ion channel proteins: detectability in sperm, preferably with knockout sperm as a negative control; ability to produce ion channel current detectable by patch-clamp recording; blocking of the channels that impairs normal sperm function; and mutations of gene encoding the ion channel proteins leading to sperm malfunctions [10].

The CatSper family of channels is the newest and only family of voltage-gated Ca^{2+} channels that meets most of the aforementioned criteria and essentially regulates Ca^{2+} entry into cells and is therefore crucial for sperm fertility [9, 45]. Four pore-forming CatSper channel proteins, CatSper 1–4, and at least two auxiliary subunits, CatSper β and CatSper γ , have been identified in a wide range of animals, including humans and mice [46, 47]. Physiologically, CatSper members are permeable to Ca^{2+} , whereas the CatSper knockdown sperm does not have the channel current that is detected in the principal piece of wild-type sperm [20, 48]. Most of the channel proteins, including CatSper members, have been identified in the principal piece of spermatozoa [20, 46, 47, 49] (Figure 1). Although the explanation of such subcellular localization is still debated, it might be because of interactions among the channel proteins and with the auxiliary subunits, although a further study is needed to resolve this issue. Collectively, these proteins play a key role in various cellular processes via regulation of the membrane potential and intracellular ionic balance. Carlson et al. [50] and Quill et al. [51] have conclusively proved that CatSper1 and CatSper2 null mice are sterile owing to their inability to generate the sperm-hyperactivated motility prerequisite for penetration of an oocyte extracellular matrix. In effect, the complete or partial absence of single or multiple Ca^{2+} channels is responsible for infertility or subfertility, although their underlying signaling cascade has not been properly studied.

Previously, it has been reported that CatSper-dependent increases of $[\text{Ca}^{2+}]_i$ in spermatozoa are induced by several psychological stimuli such as cyclic nucleotides (e.g., cAMP and cGMP) [29, 30, 52], soluble adenylyl cyclase [29, 52], zona pellucida glycoprotein [34, 35, 38], serum albumin [37, 38], secretion of cumulus oophorus [38], intracellular alkalization [3, 53], and pH [6, 21]. A recent study showed that endocrine disruptors such as p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE) promoted Ca^{2+} entry into spermatozoa by activating CatSper channels, even at a physiological concentration [36]. In addition, several other components are also known to play an

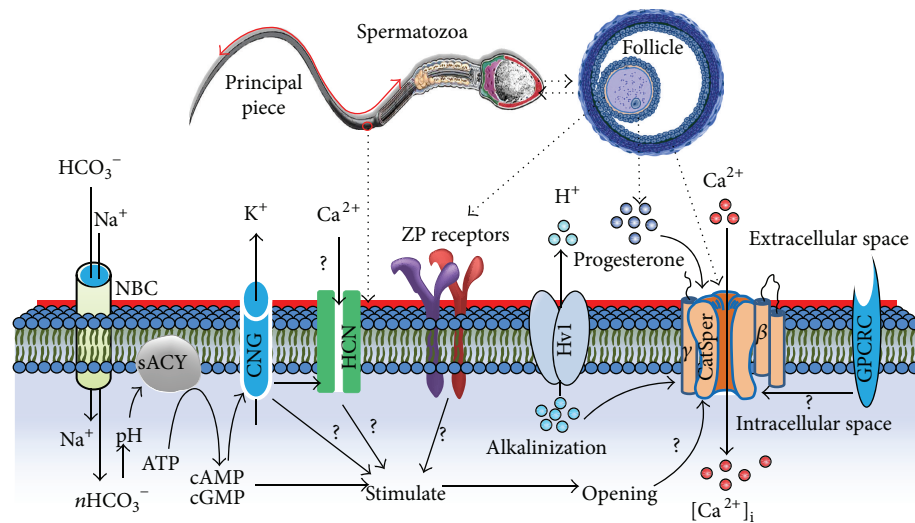


FIGURE 1: Possible signal transduction mechanisms of mammalian sperm Ca^{2+} influx through the Ca^{2+} permeable channel proteins. Previously published studies were used as references to summarize the list of channel proteins in spermatozoa. The channel proteins are localized mainly in the principle piece of spermatozoa. The follicular fluid and several factors in the fallopian tube (*in vitro* media) stimulate the receptors for spermatozoa Ca^{2+} influx. Ca^{2+} influx in spermatozoa is principally regulated by CatSper channels; however, the possible interaction between other channels that are responsible for controlling the opening of CatSper and allowing the Ca^{2+} into cells is indicated by arrow signs (red circle). The different channel proteins that are depicted in the diagram include the Na^{+} -coupled HCO_3^{-} transporter (NBC) family, soluble adenylyl cyclase (sACY), adenosine triphosphate (ATP), cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), cyclic nucleotide-gated ion channel (CNG), hyperpolarization-activated cyclic nucleotide-gated channel (HCN), zona pellucida (ZP), the voltage-gated proton channel (Hv1), glutamate receptor family class-C (GPCRC), and an unknown mechanism (?).

important role in Ca^{2+} influx mechanisms in mammalian spermatozoa by regulating the opening of CatSper members, including the flagellar voltage-gated proton channel (Hv1) [21], Ca^{2+} -ATPase pump [33], several cyclic nucleotide-gated ion channels (CNG) [27, 54], hyperpolarization-activated cyclic nucleotide-gated (HCN) channels [27], and G-protein coupled receptors (GPCRs).

A hypothetical signaling cascade of Ca^{2+} influx pathways and interaction of several channel proteins is depicted in Figure 1. Although the functions of several ion channel proteins together with their concurrent relationship with numerous stimuli have been well studied [21, 27, 38], several fundamental questions remain unanswered; for example, how do these channels/stimuli regulate the Ca^{2+} influx during spermatozoa processes such as capacitation, the acrosome reaction, and fertilization? Do they work alone or together with other channel proteins to regulate Ca^{2+} influx? Moreover, which other parameters that remain undetected could have an effect on Ca^{2+} influx? Therefore, future research should focus on resolving these issues. Table 1 summarizes the proposed effect of Ca^{2+} ion channels and their physiological role that ultimately helps Ca^{2+} influx into mammalian spermatozoa.

3. Effect of Ca^{2+} Influx on Male Fertility

Ca^{2+} triggers multiple physiological events in spermatozoa, such as hyperactivation, chemotaxis, capacitation, and the acrosomal reaction, all of which are essential for

successful fertilization. In mammalian spermatozoa, numerous Ca^{2+} permeable channel proteins control intracellular pH, and the pH-dependent Ca^{2+} influx is measured by the whole-cell patch clamp technique [9, 20]. A review of the literature showed that a potential functional interaction exists between the sperm proteins and Ca^{2+} permeable channel proteins, thus modulating the Ca^{2+} influx mechanism [4, 5, 39] and playing a vital role in adjusting male fertility. However, the mechanism by which Ca^{2+} triggers intracellular signaling to regulate physiological events in spermatozoa and the role of sperm proteins in adjustment of Ca^{2+} influx into cells remains unclear. This topic is emphasized below.

3.1. Ca^{2+} Influx, Sperm Hyperactivation, Chemotaxis, and Protein Functions. In general, mature spermatozoa are held immotile within the epididymis. However, they quickly begin to swim following release. This is known as activation of motility and is characterized by symmetrical flagellar beats [55, 56]. The terms sperm activation and hyperactivation have quite different meanings. The spermatozoa become hyperactivated when the amplitude of the flagellar bend increases and produces a highly asymmetrical beat. *In vivo*, hyperactivation of spermatozoa facilitates the release of sperm from oviductal storage and boosts them through mucus in the oviductal lumen and matrix of the cumulus oophorus during fertilization [7]. In contrast, chemotaxis is a form of sperm movement in which spermatozoa move toward a concentration gradient of a chemoattractant released from the oocyte [57, 58].

TABLE 1: Summary of published works on ion channels and physiological stimuli of mammalian spermatozoa that regulate the Ca^{2+} influx mechanism.

Name of channel/stimuli	Localization on spermatozoa/availability	Role in Ca^{2+} influx	Role in sperm physiology	Effect of knocking down/absence	Reference
CatSper CatSper 1 CatSper 2 CatSper 3 CatSper 4 CatSper β CatSper γ	Principal piece	Regulates Ca^{2+} influx	Ca^{2+} uptake, hyperactivated motility	Sterile	Barratt and Publicover, [19]; Qi et al. [20]
Hv1	Principal piece	Intracellular pH, alkalization thus stimulate Ca^{2+} influx	Extrudes protons from flagella, alkalization	Fertile	Lishko et al. [21], Lishko et al. [22]
I_{ATP}	Midpiece	Selectively transports the Ca^{2+}	Ca^{2+} influx, alkalization	Fertile	Navarro et al. [23]
TRPC	Principal piece, midpiece	Stimulates opening of CatSper	Ca^{2+} influx, cell depolarization	Fertile	Gees et al. [24], Castellano et al. [25]
CNG	Sperm flagellum, head	Stimulates opening of CatSper via cAMP/cGMP	Ca^{2+} influx	Fertile	Biel and Michalakakis [26]
HCN	Flagellum	Depolarization and opening of CatSper	Ca^{2+} influx	Fertile	Wiesner et al. [27]
SOC	Plasma membrane	ZP-induced Ca^{2+} influx	Sperm chemotactic	Subfertile	Yoshida et al. [28]
sACY cAMP/cGMP	Intracellular space and cell membrane	Activates CatSper, CNG, and HCN to regulate Ca^{2+} influx	Ca^{2+} influx, alkalization	Sterile	Esposito et al. [29], Hess et al. [30]
GPCR(s)	Principal piece, midpiece	ZP-induced Ca^{2+} influx increases in $[\text{Ca}^{2+}]_i$	Maintains fertilization	Subfertile	Fukami et al. [31] Fukami et al. [32]
PLC δ	Acrosome	ZP induced increases in $[\text{Ca}^{2+}]_i$	Ca^{2+} influx	Subfertile	Fukami et al. [32]
Ca^{2+} -ATPase pump	Principal piece	Intracellular pH and alkalization	Ca^{2+} influx, capacitation	Motility loss results in infertility	Wennemuth et al. [33]
ZP glycoproteins	Follicle	Induced Ca^{2+} -dependent increase in $[\text{Ca}^{2+}]_i$	Hyperactivation, capacitation	Delayed capacitation	Florman [34], Florman et al. [35]
Endocrine disruptor (p,p'-DDE)	Female reproductive tract	Activates CatSper	Ca^{2+} influx	Motility loss, delayed capacitation	Tavares et al. [36]
BSA	Extracellular space	Similar to ZP glycoprotein	<i>In vitro</i> capacitation	Motility loss, subfertility	Xia and Ren [37] Bailey and Storey [38]
Oviductal and follicular fluid	Extracellular space (<i>in vivo</i>)	Ca^{2+} -dependent increase in $[\text{Ca}^{2+}]_i$ in sperm	Ca^{2+} influx	Motility loss delayed capacitation	Xia and Ren [37]

Hv1: voltage-gated proton channel; I_{ATP} : ATP-gated channel; TRPC: transient receptor potential channels; CNG: cyclic nucleotide-gated ion channel; HCN: hyperpolarization-activated cyclic nucleotide-gated channel; SOC: store-operated Ca^{2+} channel; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; sACY: soluble adenylyl cyclase; GPCR: glutamate receptor family class-C; PLC δ : phospholipase C zeta; ZP: zona pellucida; p,p'-DDE: p,p'-dichlorodiphenyldichloroethylene; BSA: bovine serum albumin.

However, the molecular event that characterizes spermatozoa chemotaxis is only partially known [57].

There is strong evidence to support that sperm hyperactivation and chemotaxis are required for penetrating the zona pellucida [48, 57, 59, 60]. Incubation of spermatozoa with an extracellular Ca^{2+} source induces hyperactivation in mammalian spermatozoa [61, 62] and chemotaxis in starfish [57]. In addition, measuring cytoplasmic Ca^{2+} levels

by using the fluorescent Ca^{2+} indicator indo-1 proved that spermatozoa hyperactivation is potentially regulated by Ca^{2+} influx. However, it is unknown whether Ca^{2+} influx independently induces hyperactivation/chemotaxis in mammalian spermatozoa. Ho and Suarez [56] proposed that sperm hyperactivation induced by Ca^{2+} influx is mainly pH-dependent because sperm require a pH of 7.9–8.5 for hyperactivation, whereas activation can occur at a pH < 7.0. The proposed

model of Ca^{2+} -induced hyperactivation is represented in Figure 2.

It has recently been found by our laboratory that treatment of mouse spermatozoa with nutlin-3a, a small molecule antagonist of the mouse double minute 2 repressor, potentially downregulates the functions of the ubiquinol-cytochrome-c reductase complex component UQCRC2 and correlated with significantly reduced $[\text{Ca}^{2+}]_i$ and sperm hyperactivation. This study provided insight that the Ca^{2+} influx in spermatozoa is partially regulated by UQCRC2 protein. Kwon et al. [4] reported that blocking VDAC with 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) significantly decreased sperm hyperactivation. A significant decrease in $[\text{Ca}^{2+}]_i$ was observed in (-) DIDS conditions, while $[\text{pH}]_i$ significantly increased in (-) DIDS, regardless of Ca^{2+} . Simultaneously, a significantly elevated $[\text{pH}]_i$ was observed in (+) Ca^{2+} . This study provides strong evidence that the modulation of Ca^{2+} influx by VDACs is pH-dependent, which is consistent with the result of a previous study by Ho and Suarez [56]. Moreover, another study proposed that deamino [Cys 1, d-ArgS] vasopressin (dDAVP), an AVPR2 agonist, significantly decreased sperm motility and intracellular pH, but, interestingly, it increased $[\text{Ca}^{2+}]_i$ by regulating the function of arginine vasopressin in mice spermatozoa. However, it remains to be clarified as to why spermatozoa motility is decreased even in increased $[\text{Ca}^{2+}]_i$ conditions.

On the basis of the findings of the aforementioned studies, it is tempting to hypothesize that spermatozoa hyperactivation is mostly controlled by Ca^{2+} influx. However, potential interactions exist between protein functions. Therefore, Ca^{2+} influx, protein interaction, and hyperactivation might give numerous different annotations of upcoming research in this field. We have illustrated a schematic representation of different signaling pathways involving sperm proteins by using Pathway Studio. These proteins exhibit significant modifications to induce sperm hyperactivation and chemotaxis in spermatozoa by regulating Ca^{2+} influx (Figure 3).

3.2. Ca^{2+} Influx versus Capacitation, the Acrosomal Reaction, Fertilization, and Sperm Proteome. Mammalian fertilization is a species-specific episode that is accomplished by a complex set of molecular events. To fertilize an oocyte, multiple extreme changes occur in spermatozoa that begin from its formation in the testes of the male reproductive tract to its penetration and fusion with an egg in the female reproductive tract. Although spermatozoa are motile as well as morphologically normal after ejaculation, they are unable to fertilize an oocyte [59]. They gain the fertilization ability only after educating in the female reproductive tract [40], and the modifications that spermatozoa experience during this time are collectively known as “capacitation.” Only capacitated spermatozoa can undergo the acrosome reaction through binding to the egg zona pellucida, and they finally become capable of penetrating and fertilizing the egg [4, 18, 39].

The term “capacitation” was proposed by Austin in 1952 [1], although this concept was initially described by both Chang and Austin in 1951 [2, 41]. In fact, *in vivo* capacitation takes place in the female reproductive tract; however, it is also possible to capacitate spermatozoa *in vitro* by using particular media containing appropriate electrolytes and pH [2]. In an elegant review, Visconti summarized that the early stage of capacitation mainly comprises the bicarbonate-mediated activation of sperm motility, whereas the late stages include intracellular alkalinization, increase in protein tyrosine phosphorylation, and preparation for the acrosomal reaction [63]. These temporal differences in capacitation and the acrosome reaction require numerous mechanisms, and Ca^{2+} influx plays a significant role in the process [63, 64]. Fraser [65] reported that capacitation is a comparatively slow event that requires several hours to complete and is mainly regulated by a modest rise in $[\text{Ca}^{2+}]_i$, whereas the acrosome reaction is an exocytosis process that occurs very rapidly (within a minute) and is triggered by a large influx of $[\text{Ca}^{2+}]_i$ [65, 66].

Although the biochemical phenomenon of Ca^{2+} regulated capacitation and the acrosome reaction have been known for the last two decades, the molecular basis of this process is still poorly understood. For capacitation, the cholesterol influx initially stimulates the elevation of $[\text{Ca}^{2+}]_i$ and bicarbonate into the spermatozoa and finally activates PKA and tyrosine phosphorylation, respectively, via the production of the cAMP [66–68]. In addition, binding to the zona pellucida causes additional activation of cAMP/PKA and protein kinase C (PKC) [68–70]. Spermatozoa need $[\text{Ca}^{2+}]_i$ influx to proceed further, and they are believed to be activated by PKC through the opening of the calcium channels. Interestingly, PKA together with a secondary messenger, inositol trisphosphate, activates calcium channels localized in the outer acrosomal membrane and increases the calcium concentration in the cytosol. Further increase of cytosolic Ca^{2+} influx occurs through a store-operated calcium entry mechanism in the plasma membrane, resulting in further depletion of Ca^{2+} in the acrosome [68, 69].

In support of the aforesaid studies, several recent studies on the same topic have also hypothesized that, after the morphological maturation of spermatozoa for sperm-oocyte fusion, $[\text{Ca}^{2+}]_i$ decreases because acrosome-reacted spermatozoa release a substantial amount of Ca^{2+} from their inner cell layers [71, 72]. Ca^{2+} -mediated capacitation and the acrosome reaction have been illustrated in Figure 2 for better understanding. However, for a more in-depth understanding, we recommend reading some excellent reviews on this topic [63, 67, 73–77].

A review of the literature showed that several sperm proteins potentially regulate the Ca^{2+} -dependent capacitation and the acrosome reaction in mammalian spermatozoa [4, 5, 39]. However, how these proteins regulate the Ca^{2+} influx in spermatozoa is a matter that remains to be elucidated. Breitbart et al. [18] reported that formation of F-actin mostly depends on PKA, protein tyrosine phosphorylation, and phospholipase D activation during capacitation. Ca^{2+} is one of the principle regulators of

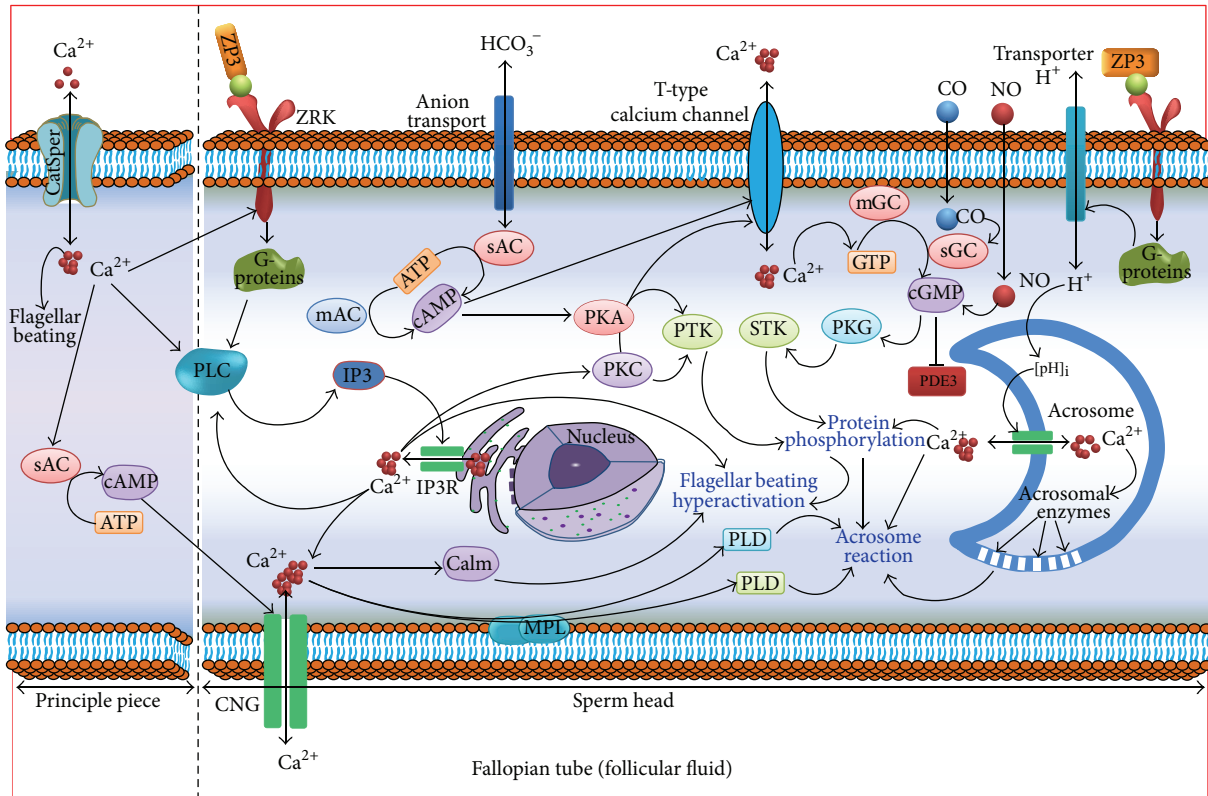


FIGURE 2: Schematic diagram showing the mechanism of Ca^{2+} regulated hyperactivation, capacitation, and the acrosome reaction of spermatozoa, which are three principal events of fertilization. Ca^{2+} together with ZP3 (zona pellucida glycoprotein-3) exhibits the most important role in sperm binding and acrosomal reaction. Ca^{2+} triggers the zona pellucida (ZP) receptors of cell membrane that activate G-proteins in the sperm head. Activated G-proteins stimulate the H^+ transporter to increase intracellular pH, ultimately inducing the acrosomal reaction and hyperactivation by catalyzing the acrosomal enzymes [91]. Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are produced from adenosine triphosphate (ATP) owing to enzymatic catalysis by soluble adenylate cyclase (sAC) and guanylate cyclase (sGC), respectively, in mature spermatozoa. The bicarbonate ions activate the sAC; however, follicular fluid also stimulates the sAC through release of Ca^{2+} ions via the CatSper channel (principal piece). However, G-protein mediated signal transduction activates sAC and phospholipase-C (PLC) that ultimately causes tyrosine phosphorylation [51, 92], which is responsible for events such as capacitation and the acrosomal reaction. Likewise, extracellular signals such as nitric oxide (NO) and carbon monoxide (CO) stimulate membrane-bound GC (mGC) and sGC, respectively, to synthesize cGMP. Increases in cGMP level evoke a concomitant increase in cAMP by inhibiting its PDE3. However, the increased Ca^{2+} level can also directly catalyze cAMP [93, 94]. Activated sAC, sGC, and PLC stimulate the generation of the second messengers' inositol trisphosphate (IP3) like cAMP, cGMP. The IP3 binds to the IP3 receptor (IP3R) to increase $[\text{Ca}^{2+}]_i$ via the release of the $[\text{Ca}^{2+}]_i$ storage ions. Concurrently, the second messengers activate protein kinases (PKA, PKC, and PKG), in turn gating ions through the T-type calcium channels, cyclic-nucleotide gated ion channel (CNG), and so on, that together with the activation of protein tyrosine kinases (PTK) and serine/threonine protein kinase (STK) cause increased protein phosphorylation [93, 94]. Additionally, the CatSper Ca^{2+} activates calmodulin (Calm), phospholipase-A (PLA), and phospholipase-D (PLD) with increased generation of other second messengers during the acrosome reaction. Ca^{2+} influx together with increased protein phosphorylation brings about the capacitation response that is responsible for the waveform asymmetry of motility termed hyperactivation during fertilization. Both hyperactivation and the acrosomal reaction boost flagellar beating, ultimately resulting in the penetration of the outer egg coat and subsequent fertilization of the mature ovum [91-95].

capacitation, and it is therefore tempting to hypothesize that organizational modification of F-actin in spermatozoa together with interacting with other sperm proteins has potential influence on Ca^{2+} influx. A similar finding has been established more precisely by another study [78], where boar sperm capacitation was studied by combined application of computational and experimental approaches. These authors reported that the boar spermatozoa capacitation network contains several connecting cascades, whereas only three nodes bound to all the subcellular compartments are

involved in spermatozoa postejaculatory signaling, such as $[\text{Ca}^{2+}]_i$, ATP, and actin polymerization. Removal of the actin polymerization node from this aforesaid network causes disorganization of the network topography and affects capacitation, and this has been confirmed by zona pellucida-induced capacitation and the acrosomal reaction in an *in vitro* demonstration [78].

In another study, Patrat et al. [79] showed that progesterone (P_4) that is secreted by cumulus cells directly acts on the sperm plasma membrane and triggers the

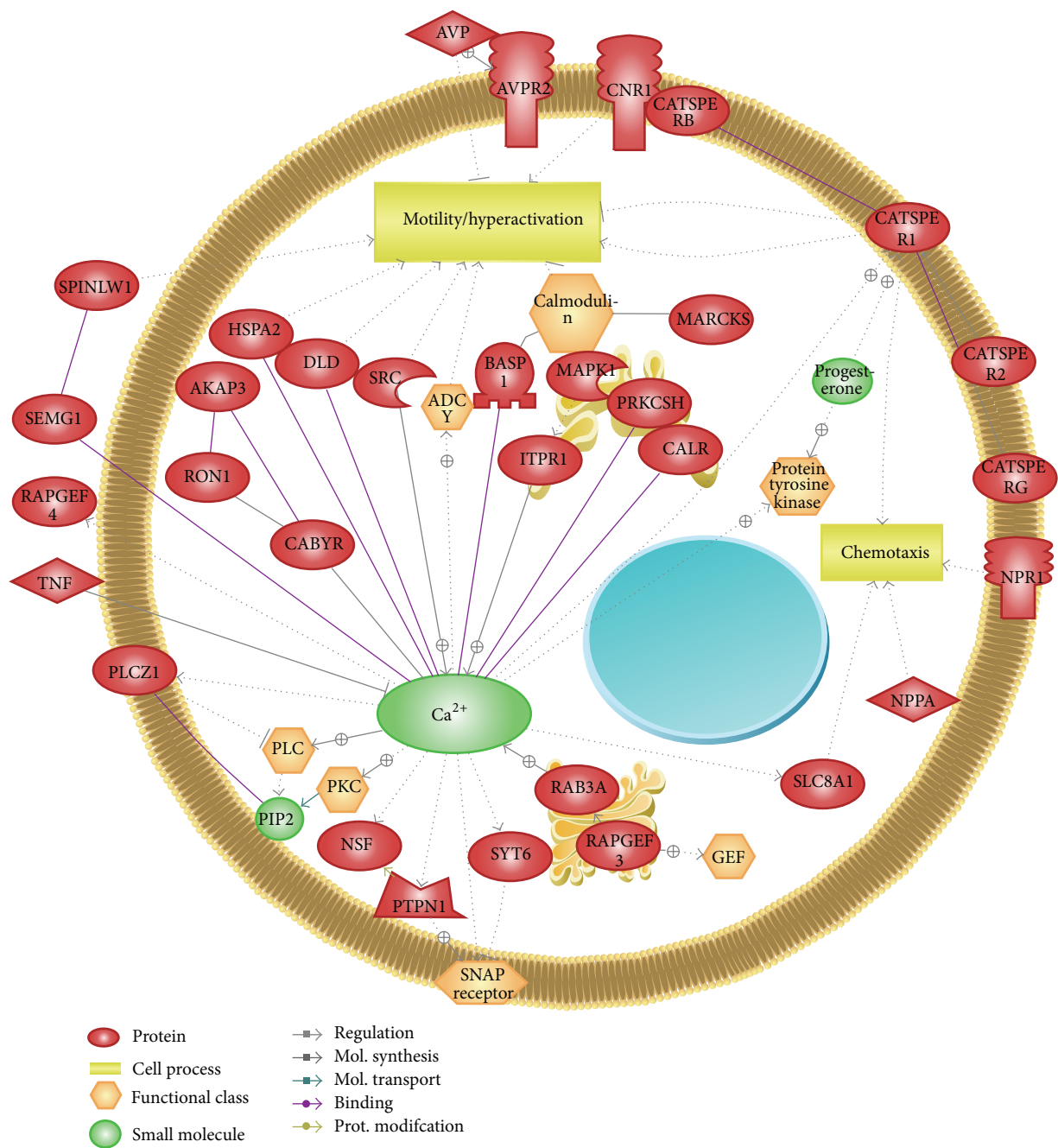


FIGURE 3: Schematic representation of interactions among ~35 proteins related to Ca^{2+} regulated spermatozoa hyperactivation and chemotaxis. The figure was produced by use of Pathway Studio (9.0) following the MedScan Reader (5.0) protein search from PubMed database [12].

intracellular signals and enzymatic pathways involved in the acrosome reaction. P_4 regulates the acrosome reaction and is mediated by a compulsory Ca^{2+} increase. This study found that P_4 induced the activation of G_i/G_o protein-coupled and protein tyrosine kinase receptors, and it affected capacitation and the acrosome reaction. In contrast, Ca^{2+} regulated exocytosis of spermatozoa requires active acrosomal proteins such as N-ethylmaleimide-sensitive factor (NSF) [66]. Additionally, the same research team showed that the

ras-related protein Rab-3A (RAB3A) is also necessary for Ca^{2+} -dependent exocytosis. Interestingly, Rab3A activation of acrosomal exocytosis requires active NSF. Therefore, protein-protein interaction might also play a potential role in regulating Ca^{2+} influx. All of these observations seem to be consistent with the idea that Ca^{2+} functions are regulated by sperm proteins during fertilization. However, the key question is how do these proteins modify Ca^{2+} influx in spermatozoa?

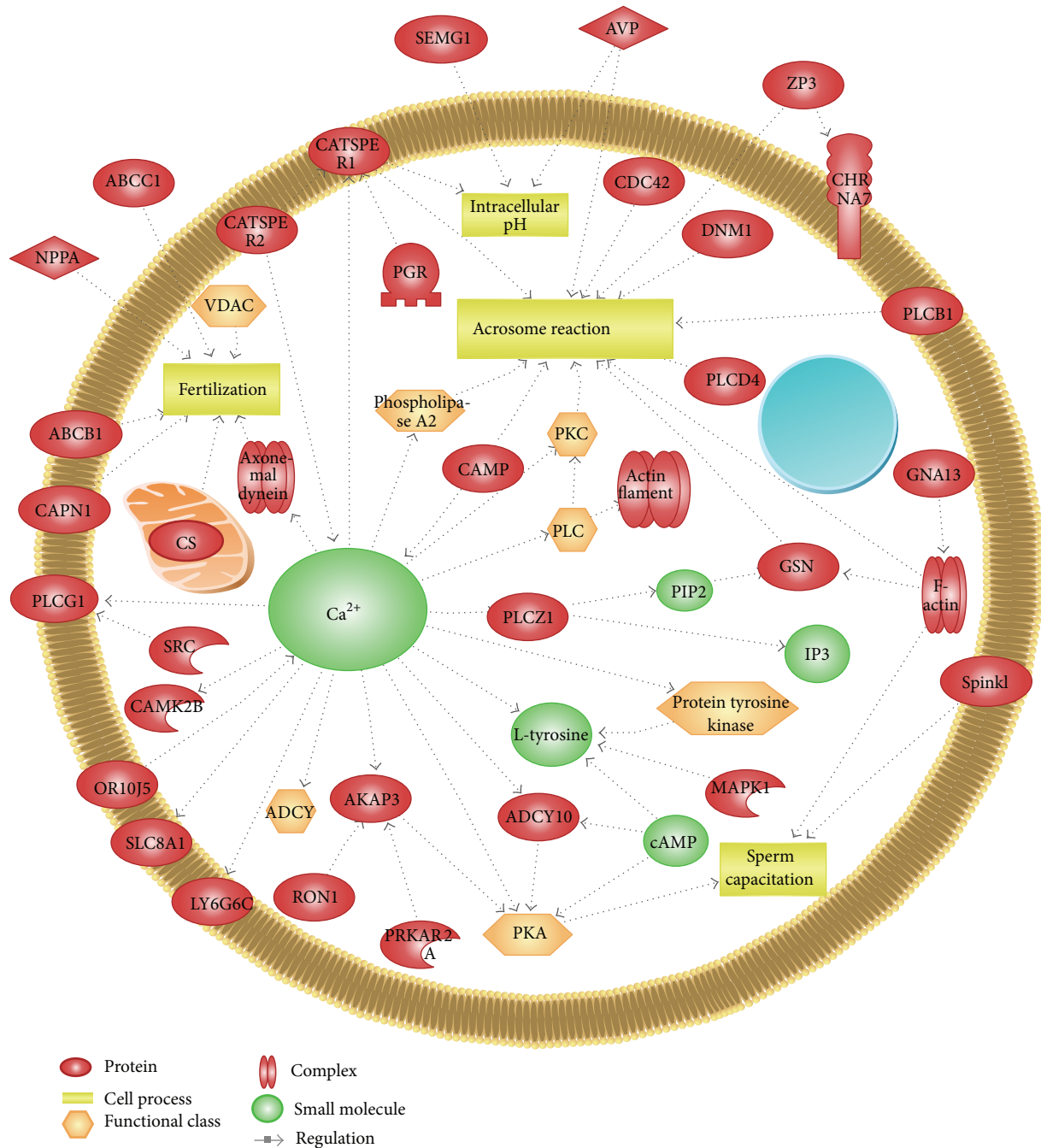


FIGURE 4: Schematic representation of interactions among ~40 proteins related to Ca^{2+} regulated spermatozoa capacitation, the acrosome reaction, and fertilization. The figure was produced by use of Pathway Studio (9.0) following the MedScan Reader (5.0) protein search from PubMed database [12].

Recently, in our laboratory, we used mice spermatozoa to evaluate the interrelationship of proteins related to Ca^{2+} influx, including UQCRC2 [39], arginine vasopressin [5], and VDACs [4], and evaluate their effects on capacitation and the acrosome reaction. It is likely that a sustained phase of Ca^{2+} is required for fertilization and might be regulated by the complex interaction of numerous sperm proteins. Therefore, studies to identify proteins that might have the ability to induce such a change are worth undertaking. Application

of Pathway Studio helped us represent over 40 proteins that are potentially implicated in Ca^{2+} mediated regulation of capacitation, the acrosome reaction, and male fertility (Figure 4).

3.3. Ca^{2+} Influx and Postfertilization Egg Activation in Context of Sperm Proteome. Ca^{2+} influx in spermatozoa is not only important for sperm maturation, but it is also equally

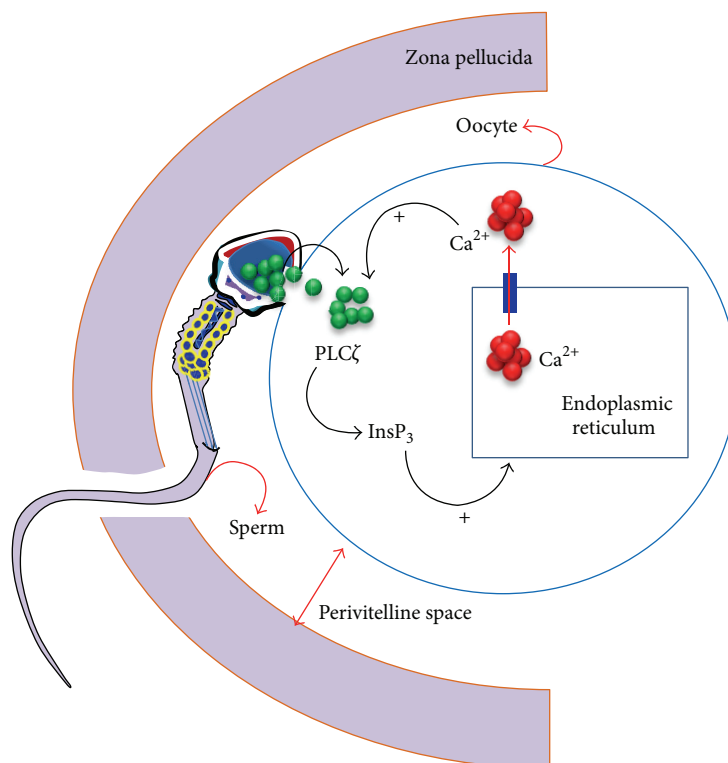


FIGURE 5: Schematic representation showing the Ca^{2+} influx mechanism in mammalian eggs stimulated by mature spermatozoa. Spermatozoa donate the phospholipase C isoform zeta ($\text{PLC}\zeta$) protein within a few minutes of sperm-egg fusion (represented by green color circle). Inositol 1,4,5-trisphosphate (InsP_3) is produced due to the hydrolysis of $\text{PLC}\zeta$, which subsequently triggers the InsP_3 receptor-mediated Ca^{2+} release (indicated by red color circle) from the endoplasmic reticulum of the oocyte. Simultaneously, the increased cytoplasmic Ca^{2+} leads to further $\text{PLC}\zeta$ stimulation, leading to the positive feedback loop of Ca^{2+} and InsP_3 rise. The hypothesis has been modified from Swann [96] and Swann et al. [97].

important for activation and development of the oocyte. A study of egg activation by Ca^{2+} was conducted by Steinhardt and colleagues in 1974 and showed remarkable findings [80]. Steinhardt et al. [80] reported that administration of Ca^{2+} ionophores induced the early events of hamster egg activation. Thus far, it has been shown that the eggs of almost all species are activated by an increase in Ca^{2+} oscillation by spermatozoa during fertilization [81, 82]. However, how the spermatozoa trigger the oocyte Ca^{2+} oscillation remains to be elucidated. Several hypotheses have been proposed to describe these mechanisms [83–86].

It has been reported previously [83] that the spermatozoa introduce Ca^{2+} influx into oocytes by a specific protein called oscilligen in hamsters. Recent studies have shown that phospholipase C zeta ($\text{PLC}\zeta$), a novel sperm-specific agent, is responsible for induction of Ca^{2+} oscillation in eggs after sperm-egg membrane fusion [87–89]. According to this mechanism, the sperm protein $\text{PLC}\zeta$ causes the release of $[\text{Ca}^{2+}]_i$ in eggs and is mediated via inositol 1,4,5-trisphosphate (InsP_3) receptors (hypothetical depiction in Figure 5). Even when the InsP_3 or its derivatives are injected into unfertilized, mature eggs, oscillation occurs due to the unique feedback properties of the InsP_3 receptors in mouse eggs [90]. However, it is still unknown whether there are any other factors/proteins available in spermatozoa that also

have similar effects. We illustrated the relevant signaling and metabolic pathways by using sperm proteins to facilitate the understanding of the mechanisms behind Ca^{2+} mediated activation of oocytes (Figure 6).

4. Future Prospects

The maturational events of mammalian spermatozoa are strictly regulated through the well-coordinated Ca^{2+} influx. It is the central regulator of many key activities in spermatozoa, all of which are necessary for fertilization. However, our current understanding at the molecular level concerning Ca^{2+} signaling in the spermatozoa is insufficient. Therefore, a better understanding of such an event can provide a more complete comprehension of Ca^{2+} regulated sperm functions and fertility optimization.

A large number of Ca^{2+} permeable ion channel proteins have been identified [10, 43] that collectively regulate the Ca^{2+} influx mechanism in spermatozoa. Although the recent application of patch-clamp recordings of channel current significantly improves our understanding of the functions of these channel proteins, several basic aspects remain unsolved, such as identifying the functions of individual channels in spermatozoa and how these channels coordinate Ca^{2+} influx.

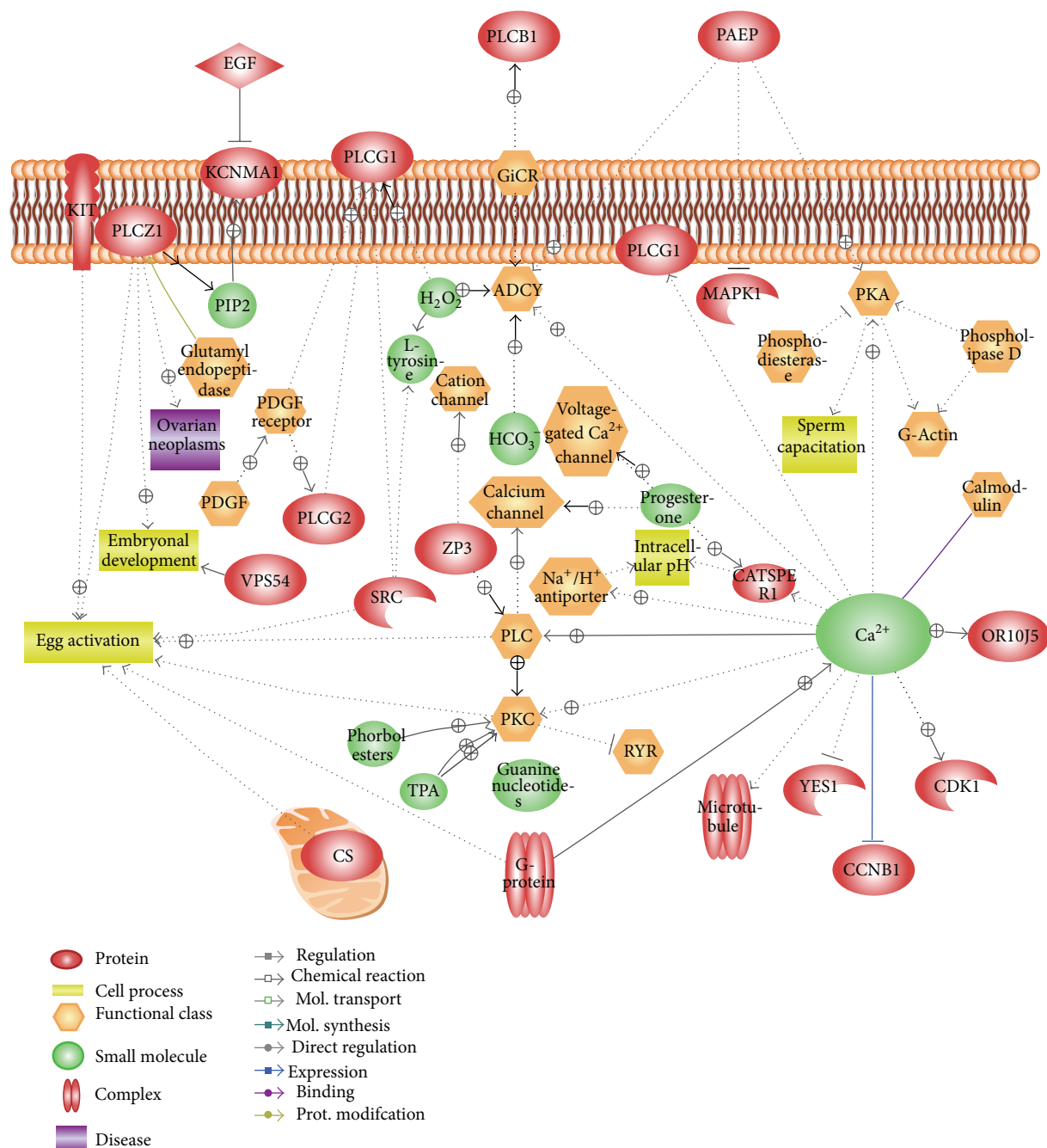


FIGURE 6: Schematic representation of interactions among ~30 proteins related to Ca^{2+} regulated egg activation and embryonic development. The figure was produced by the use of Pathway Studio (9.0) following the MedScan Reader (5.0) protein search from PubMed database [12].

Therefore, production of knockdown animals and using them as negative controls compared with their wild counterparts might provide more specific ideas about channel functions. CatSper is the one of the well-studied channel proteins [10] and the functions of different pore-forming CatSper channels (1–4) and auxiliary subunits (CatSper β and CatSper γ) remain a matter to be elucidated.

A literature review found that the Ca^{2+} influx mechanism in spermatozoa is regulated by several physical stimuli,

although the underlying mechanism is less clearly defined. Protein-protein interactions also potentially regulate the Ca^{2+} uptake mechanism in spermatozoa. Although recently applied proteomic approaches have identified several sperm-specific proteins, their functions in Ca^{2+} regulation and interaction with channel proteins are unclear. Therefore, future research should target this topic to provide a robust understanding of Ca^{2+} and male fertility in both humans and other animal species.

Conflict of Interests

The authors have declared there is no conflict of interests that could be perceived as prejudicing the impartiality of this paper.

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Review Article

Stem Cells as New Agents for the Treatment of Infertility: Current and Future Perspectives and Challenges

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Stem cells are undifferentiated cells that are present in the embryonic, fetal, and adult stages of life and give rise to differentiated cells that make up the building blocks of tissue and organs. Due to their unlimited source and high differentiation potential, stem cells are considered as potentially new therapeutic agents for the treatment of infertility. Stem cells could be stimulated *in vitro* to develop various numbers of specialized cells including male and female gametes suggesting their potential use in reproductive medicine. During past few years a considerable progress in the derivation of male germ cells from pluripotent stem cells has been made. In addition, stem cell-based strategies for ovarian regeneration and oocyte production have been proposed as future clinical therapies for treating infertility in women. In this review, we summarized current knowledge and present future perspectives and challenges regarding the use of stem cells in reproductive medicine.

1. Introduction

Nearly 72.4 million people or 15% of couples experience fertility problems [1]. For couples and clinicians, a diagnosis of infertility signals the start of investigations and possible treatment. Infertility, defined as failure to conceive a clinically detectable pregnancy after >12 months of unprotected intercourse, is a common condition, reported by 1 in 6 couples [1, 2]. As infertility is a heterogeneous condition, caused by various underlying pathologies, it is possible that some of the mechanisms leading to infertility also play a role in the etiology of this outcome [3–5]. In recent years, several advancements have been made in assisted reproduction treatment and now more than 80% of couples experiencing infertility issues can conceive a child [6].

Due to their unlimited source and high differentiation potential, stem cells are considered as potentially new therapeutic agents for the treatment of infertility. In this review,

we will summarize current knowledge regarding the use of stem cells in reproductive medicine.

2. Stem Cells: A Novel Hope in Cell-Based Therapy

Stem cells are undifferentiated cells that are present in the embryonic, fetal, and adult stages of life and give rise to differentiated cells that are building blocks of tissue and organs (Table 1). In the postnatal and adult stages of life, tissue-specific stem cells are found in differentiated organs and are instrumental in repair following injury to the organ. The major characteristics of stem cells are (a) self-renewal (the ability to extensively proliferate), (b) clonality (usually arising from a single cell), and (c) potency (the ability to differentiate into different cell types) [7, 8]. Totipotent or omnipotent cells are the most undifferentiated cells and

TABLE 1: Characteristics of stem cells used in stem cell-based therapy of infertility.

ESCs	MSCs	Stem cell from extraembryonic tissues	iPSCs	Spermatogonial stem cells
Derived from inner cell mass of the blastocyst	Derived from bone marrow, adipose tissues, bone, Wharton's jelly, umbilical cord blood, and peripheral blood	Derived from amnion, chorion, placenta, and umbilical cord	Derived from somatic cells	Derived from testicular tissues
Pluripotent	Multipotent	Multipotent	Pluripotent	Pluripotent
These cells can differentiate into cell types of all three germ layers	These cells can differentiate into mesoderm-derived tissues (adipose tissues, bone, cartilage, and muscle)	These cells can differentiate into adipocytes, endothelial cells, hepatocytes, osteocytes, myocytes, and neurons	These cells can differentiate into cell types of all three germ layers	These cells can differentiate into cell types of all three germ layers
Prolonged proliferation	Degree of proliferation depends on the tissue from which these cells were isolated	Degree of proliferation depends on the tissue from which these cells were isolated	Prolonged proliferation	Difficult to be maintained in cultures
Indefinite self-renewal potential	Limited self-renewal	Limited self-renewal	Indefinite self-renewal potential	Self-renewal ability to go through numerous cell divisions while maintaining the undifferentiated state
High telomerase activity	Low telomerase activity	Low telomerase activity	High telomerase activity	High telomerase activity
Immortal; cell lines remain intact for long periods of time and produce endless numbers of cells	Production of limited number of cells	Production of limited number of cells	Immortal; cell lines remain intact for long periods of time and produce endless numbers of cells	—
These cells are not immune privileged	These cells have immunomodulatory characteristics	—	These cells are not immune privileged	These cells are not immune privileged

are found in early development. A fertilized oocyte and the cells of the first two divisions are totipotent cells, as they differentiate into both embryonic and extraembryonic tissues, thereby forming the embryo and the placenta [9]. Pluripotent stem cells are able to differentiate into cells that arise from the 3 germ layers—ectoderm, endoderm, and mesoderm—from which all tissues and organs develop [10]. Commonly, stem cells are derived from two main sources: early embryos (embryonic stem cells (ESCs)) and adult tissue (adult stem cells).

ESCs are pluripotent stem cells derived from the inner cell mass of the blastocyst [11]. The essential characteristics of ESCs include derivation from the preimplantation embryo, prolonged proliferation in their pluripotent state, and stable developmental potential to form derivatives of all three embryonic germ layers [11].

Mesenchymal stem cells (MSCs) are one of the most common adult, multipotent stem cells [12]. They can be derived from a variety of tissues including bone marrow, adipose tissue, bone, Wharton's jelly, umbilical cord blood, and peripheral blood [13]. MSCs are adherent to cell culture dishes and are characterized by specific surface cell markers. MSCs show variable levels of expression of several molecules, CD105 (SH2), CD73 (SH3/4), stromal antigen 1, CD44, CD166

(vascular cell adhesion molecule), CD54/CD102 (intracellular adhesion molecule), and CD49 (very late antigen), and lack the expression of surface markers characteristic for HSCs (CD14, CD34, CD45, and CD11a/LFA-1), erythrocytes (glycophorin A), and platelet and endothelial cell (CD31). MSCs are able to differentiate into mesoderm-derived tissue such as adipose tissue, bone, cartilage, and muscle [13–16]. Recently, MSCs were differentiated into neuronal tissue which is derived from the ectoderm. This is an example of transdifferentiation, that is, when a cell from one germ layer (mesoderm) differentiates into neuronal tissue (ectoderm) [17].

Stem cells can also be derived from extraembryonic tissues (amnion, chorion, placenta, and umbilical cord) [18]. Amnion and chorion contain stromal cells that display characteristics and differentiation potential similar to bone marrow-derived MSCs and are able to differentiate into adipocytes, endothelial cells, hepatocytes, osteocytes, myocytes, and neurons [7, 18]. Placental-derived stem cells have the capacity to differentiate into ectodermal, mesodermal, and endodermal cell types, while umbilical cord matrix stem cells, after transplantation, enhanced muscle regeneration in mouse model of severe muscle damage and promoted blood vessel formation and neurological function

TABLE 2: Potential advantages and disadvantages of stem cells in regenerative medicine.

Stem cells	Advantages	Disadvantages
ESCs	Pluripotent; high telomerase activity	Ethical concerns; malignant potential; difficult to control; may require many steps to differentiate into desired cell type; immune rejection
MSCs	No ethical or moral concerns; low malignant potential; avoiding allogeneic immune rejection	Limited flexibility; multipotent; difficulty to be maintained in cell culture for long periods
Stem cell from extraembryonic tissues	No ethical or moral concerns; reducing risk of tumorigenicity	Limited flexibility; multipotent
iPSCs	No ethical or moral concerns; patient-specific cells	Use of viral vectors to introduce genes; malignant potential
Spermatogonial stem cells	No ethical or moral concerns	Relatively small numbers in testis; difficulty to be maintained in cultures; immune rejection

in animal models of ischaemic brain disease [18]. The main advantage of stem cells derived from extraembryonic tissues is the efficient isolation from tissues normally discarded at birth avoiding ethical concerns that plague the isolation of human embryonic stem cells [7] (Table 2).

Recently, Takahashi and Yamanaka [19] generated pluripotent cells by reprogramming somatic cells. These cells are called induced pluripotent stem cells (iPSCs) and share similar characteristics with ESCs: exhibiting morphology of ESCs, expressing ESCs markers, having normal karyotype, expressing telomerase activity, and maintaining the developmental potential to differentiate into derivatives of all three primary germ layers. Thus, iPSCs are adult cells that have been genetically reprogrammed to an embryonic stem cell-like state by being forced to express genes and factors important for maintaining the defining properties of embryonic stem cells [20]. Transplantation of stem cells or their derivatives into respective tissues or organs is considered as one of the most promising remedies for many incurable diseases. Unfortunately, immune compatible cells are hardly obtainable for any given patient because of the specificity and complexity of human immune system. In this regard, induced pluripotent stem cells (iPSCs) and gene editing technologies are believed to offer an unprecedented solution for obtaining sufficient healthy autologous cells [21]. However, it should be emphasized that, despite numerous technical advances in the reprogramming technology, iPSCs apart from a very small number of ongoing clinical studies are not yet ready for transplanting into patients. Relatively little is known about iPSCs molecular and functional equivalence to hESCs and careful analysis of the genomic and epigenomic integrity of human iPSCs is required before their therapeutic use.

Stem cells could be stimulated *in vitro* to develop various numbers of specialized cells including male and female gametes suggesting their potential use in reproductive medicine.

3. Stem Cells Are Novel and Unlimited Source for Male Gametes: True or False?

During past few years a considerable progress in the derivation of male germ cells from pluripotent stem cells has been

made [22–24]. These studies provide a desirable experimental model for elucidating underlying molecular mechanism of male germ cell development and potential strategies for producing haploid germ cells for the treatment of male infertility.

Spermatogenesis is a complex process by which spermatogonial stem cells (SSC) self-renew and differentiate into haploid spermatozoa. In mammals, this process takes place in the seminiferous tubules of testis, which provide a functional niche for male germ cells [25] and involve three major stages: mitosis, meiosis, and spermiogenesis [24]. Errors at any stage of spermatogenesis can result in subfertility and infertility [26].

SSC reside in adult testis and maintain spermatogenesis and continual sperm production throughout a male's lifespan [27]. SSC are diploid cells that originate from less differentiated primordial germ cells that migrate to the gonadal ridges during embryogenesis [28]. SSC can be found in the seminiferous tubule, lying near to the basement membrane [29]. Several markers could be used for the identification and isolation of SSC: spermatogonia-specific marker Stra8 for mouse SSC [30, 31], thymocyte antigen 1 (Thy-1), CD9, stage-specific embryonic antigen-4 (SSEA4), $\beta 1$ and $\alpha 6$ integrins for rat SSC [32], SSEA4, and G-protein coupled receptor 125 (GPR125) for human SSC [33]. SSC are a potential tool for the treatment of male infertility due to their ability to differentiate into male gametes *in vitro* and capacity to restore male fertility *in vivo* [34, 35]. SSC are adult stem cells, but SSC-derived cells, called multipotent adult germline stem cells (maGSC), have differentiation potential similar to ESCs. *In vitro*, maGSC are able to spontaneously differentiate into derivatives of all embryonic germ layers and are able to generate teratomas after transplantation in immunodeficient mice [31]. Nolte and coworkers showed that maGSC are able to undergo meiosis and form haploid male germ cells *in vitro* [30]. An important breakthrough for SSC-mediated spermatogenesis was made by Hermann and coworkers [35]. They showed that autologous and allogeneic SSC transplantations into the testes of adult and prepubertal recipient macaques, which were rendered infertile with alkylating chemotherapy, regenerate spermatogenesis resulting in production of functional sperm. These results strongly indicate SSC transplantation as a novel and successful therapeutic tool for male infertility caused

by chemotherapy before puberty [35]. Although SSC seem to be a good candidate for the stem cell-based therapy of male infertility, a low concentration of SSCs in mammal testis and challenges associated with protocols for their isolation, identification, and culturing have to be addressed before their clinical use [29].

Hübner et al. first reported the successful derivation of gametes from mouse embryonic stem cells (ESCs) *in vitro* [36]. Afterwards, different studies with mouse ESCs have shown the ability to make functional spermatozoa [37, 38] capable of giving rise to live offspring after use of intracytoplasmic injection [37]. Differentiation of male germ cells from human ESC has also been demonstrated [39–43]. Similarly, studies with human ESCs have revealed the ability to differentiate *in vitro* into advanced spermatogenic stages, including round spermatids which are not capable of fertilizing oocytes in high-order mammals [22, 23].

Besides the fact that ESCs are genetically unrelated to the patient in need of fertilization treatment, the isolation of human ESCs is ethically controversial because it involves the destruction of human embryos. The significant breakthrough in stem cell biology, a discovery of patient-specific induced pluripotent stem cells (iPSCs), may overcome these issues. Recently, several studies have reported that both mouse iPSCs [24, 44, 45] and human iPSCs can differentiate into male germ cells [20, 21].

It has been verified that mouse iPSCs can form functional spermatozoa [46, 47]. Functional assays have shown that spermatozoa generated from iPSCs were capable of fertilizing the oocytes after intracytoplasmic injection and giving rise to fertile offspring following embryo transfer [46]. So far, functional male gametes from human iPSCs have not been obtained.

There are two possible approaches in generating of male germ cells from pluripotent stem cells: *in vitro* differentiation into advanced, haploid cell products [20, 21] or combined *in vitro* differentiation and *in vivo* transplantation [24, 48]. Generally, there are two methods to produce male gametes from the pluripotent stem cells *in vitro* [20]: the monolayer differentiation and the embryoid body (EB) formation [24, 44]. Direct differentiation on monolayers of human fibroblast ensures more consistent differentiation results compared with EB formation [20].

Different growth factors or cytokines could induce pluripotent stem cells into germ cells *in vitro* (Figure 1), such as bone morphogenetic protein 4, stem cell factor, epidermal growth factor, and forskolin, but most of *in vitro* differentiation protocols include retinoic acid (RA) induction [46, 49, 50]. It has been shown that RA, an active derivate of vitamin A, regulates the timing of meiotic initiation in mice [50, 51]. Some protocols include combination of RA and testosterone [45] or subsequent exposure to differentiation cocktail containing forskolin, human leukemia inhibiting factor (LIF), bFGF, and CYP26 inhibitor R115866 [22]. Testosterone is required for spermatogenesis *in vivo* and stimulates Sertoli cells to produce different growth factors, including stem cell factor that promotes germ cell differentiation [52]. Forskolin is involved in meiosis induction [53] and induces germ cell proliferation by activation of cyclic

adenosine monophosphate [54]. LIF promotes survival and proliferation of gonocytes [55], while bFGF helps balancing self-renewal and differentiation of SSC [56]. R115866 acts by suppressing the inhibitory effects of CYP26 on STRA8, the meiosis regulator gene [51].

In some studies combination of *in vitro* differentiation followed by *in vivo* transplantation was performed in order to gain male gametes in advanced differentiation stages [24, 48] (Figure 1). Most often, pluripotent stem cells are induced in SSC-like stages *in vitro* and then transplanted into sterile mice testis [24] or ectopic, into the dorsal region of the mice, together with immature testicular cell suspension [44, 48]. It has been shown in several animal models that SSC-like cells are capable of recolonizing the testis [34, 35] and exhibit proper spermatogenesis [46]. A limiting step for stem cell replacement therapy of infertility could be the damaged somatic environment of the testis. If the somatic environment is damaged it is not receptive to SSC transplantation and thereby not able to restore patient fertility [57]. Ectopic cotransplantation of SSC with testicular cells might be a way to overcome this limitation but Yang et al. have reported that, although iPSC-derived germ cells could reconstitute seminiferous tubules and settle at basement membrane, no further differentiation was observed in reconstituted seminiferous tubules [44].

4. Stem Cell-Derived Oocytes: Current Knowledge and Future Perspectives

Stem cell-based strategies for ovarian regeneration and oocyte production have been proposed as future clinical therapies for treating infertility in women.

There has been a long-persisting dilemma regarding the presence of ovarian stem cells in adult mammalian ovaries. Several research studies claimed that they have identified functional oogonial stem cells in the postnatal ovary of several different species including humans and now there is steadily increasing experimental evidence on their existence [58]. An important breakthrough was made by Zou and his coworkers who successfully established long-persisting pluripotent/multipotent ovarian stem cell lines in neonatal and adult mice [59]. They detected cells residing within the ovarian surface epithelium of neonatal and adult mice that were double positive for mouse vasa homologue (MVH) and DNA marker 5-bromodeoxyuridine (BrdU) confirming that these cells were of germ cell lineage and exhibited a replicative potential (Figure 2). With passage in culture, the cells isolated by Zou et al. were confirmed to have significant proliferative capacity and expressed high telomerase activity, *Oct4*, and *Nanog*. The cells were then marked using a retroviral vector bearing green fluorescent protein (GFP) before being directly delivered into the ovaries of adult female mice rendered sterile by treatment with chemotherapy. Importantly, GFP⁺ follicles in various stages of maturation were observed several weeks later in the ovaries of the conditioned mice indicating that isolated ovarian stem cells were capable of regenerating functional oocytes when transplanted back into sterile recipient mice [59] (Figure 2).

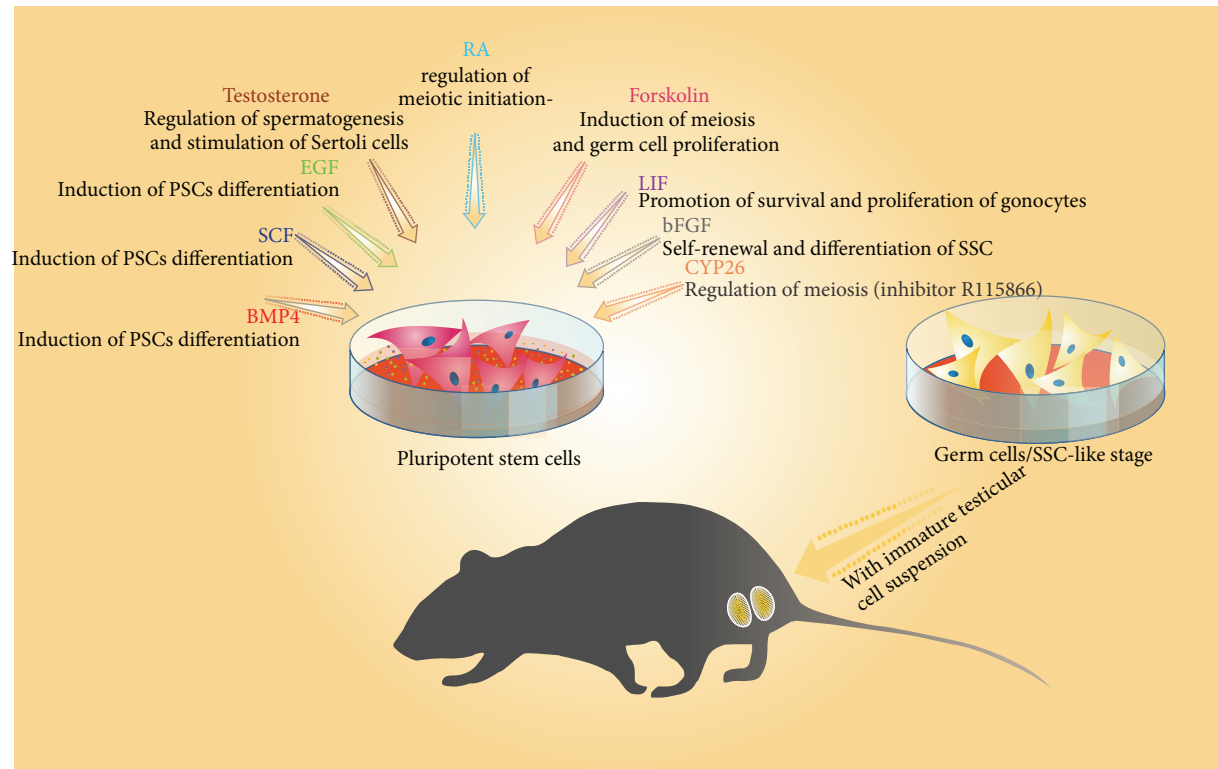


FIGURE 1: Stem cell-derived male gametes. Several growth factors and cytokines are used for *in vitro* differentiation of pluripotent cells into male gametes/SSC-like cells. The transplantation of stem cell-derived SSC-like cells in sterile mice results in proper spermatogenesis.

Recently, the work by White et al. has identified a rare population of mitotically active germ cells in human ovaries that can be purified and cultured *in vitro* to spontaneously form oocytes [60]. These cells, named as germ stem cells (GSCs), were isolated from reproductive-aged human ovaries using fluorescence-activated cell sorting (FACS) with an antibody against the carboxyl (–COOH) terminus of the germ cell-specific marker Ddx4, which is expressed on the cell surface of GSCs. Further, GSCs were capable of forming oocyte-like structures and incorporating into follicles under specific *in vitro* and *in vivo* conditions. This work highlights a unique potential to generate oocytes *in vitro* from isolated cells in reproductive-aged women who may have a depleted follicle pool from such genetic defects as fragile X-associated primary ovarian insufficiency. This recent advance, along with those described above, highlights the unique methodologies being developed to combat female-factor infertility representing a significant step towards the revolutionary idea of neo-oogenesis in reproductive-aged women through the isolation and characterization of germ stem cells.

However, despite the discovery of the potential germ stem cells in mammalian ovaries, it remains uncertain whether these cells exist and function in ovaries under physiological conditions. Liu et al. concluded that active meiosis, neo-oogenesis, and GSCs are unlikely to exist in normal, adult human ovaries [61]. Findings published by Zhang et al. contradict the results obtained by White et al. and Zhang et al. used fluorescent proteins to identify GSCs in the ovaries

of mice, but these cells failed to divide or differentiate into oocytes [62]. The scientific community has questioned both the methods and significance of these studies. Supporters of postnatal de novo oogenesis disagree with the study conducted by Zhang et al. and state that the study investigated oocytes and not GSCs in their applied experimental setting; thus, the researchers never observed mitosis in Ddx4-positive cells since oocytes expressing cytoplasmic Ddx4 do not divide [63]. According to White et al., Ddx4 is found on the cell surface of GSCs and thus enables FACS-based isolation of living GSCs from adult mouse and human ovaries [60]. This is in contrast to the opinion of Zhang et al., who argue that Ddx4 is expressed only in the cytoplasm and not on the cell surface and hence FACS-based isolation of GSCs is problematic [64]. While the debate continues, only future experiments will help to clarify this issue.

In the meantime, several studies were published regarding the potential of pluripotent stem cells for differentiation into oocytes. Eguizabal and coworkers managed to generate haploid female cells from human pluripotent stem cells, but neither of them resembled an oocyte nor is predicated to possess a functional ooplasm capable of being fertilized [22]. However, the recent work by Hayashi et al. showed that mouse stem cells could be differentiated in an *in-vitro/in-vivo* system into oocyte-like cells that are capable of being fertilized by spermatozoa and generating normal progeny [65]. This outstanding advancement further shows the ability of pluripotent stem cells to differentiate into all cells of the

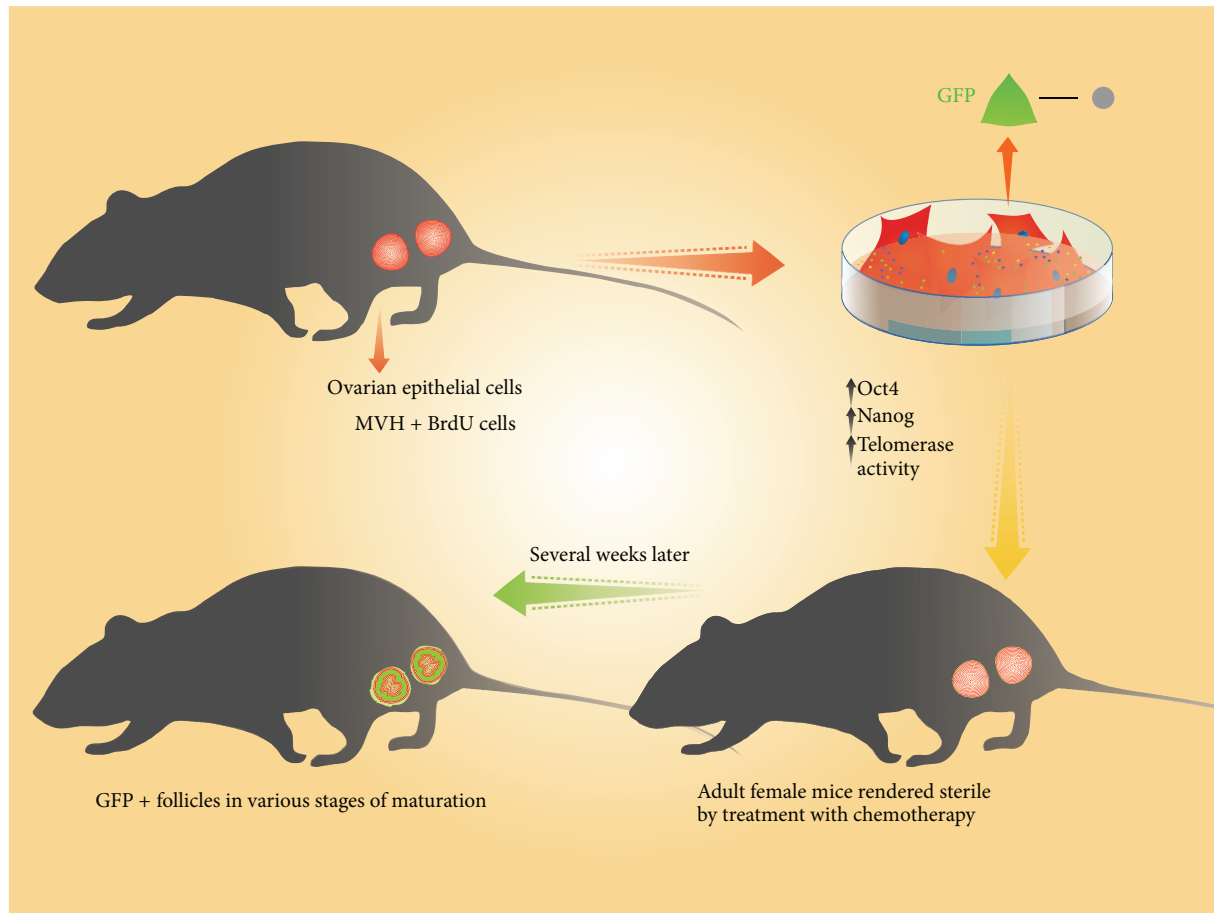


FIGURE 2: Ovarian stem cells: isolation and regenerative potential. Ovarian stem cells (MVH+BrdU+ cells) residing within the ovarian surface epithelium of neonatal and adult mice express high telomerase activity, Oct4, and Nanog and have a capacity to generate functional oocytes when transplanted back into sterile recipient mice.

adult organism [64]. Whether the work by Hayashi and colleagues can be adapted for human stem cells remains to be seen, but this advancement is a critical step forward in generating functional de novo oocytes from human iPSCs obtained from female patients rendered sterile by medical interventions, exposure to toxicants, or premature ovarian failure [64].

5. Conclusions

Pluripotent stem cells open new perspectives in the treatment of patients with azoospermia. Although the use of ESCs is connected with many ethical concerns, there are no ethical issues regarding the use of iPSCs. Moreover, ESCs are genetically unrelated to the patients, while it may be possible to get offspring with their own genetics by using iPSCs in derivation of functional male gametes.

The potential clinical applications of putative ovarian derived stem cells are apparent. The development of techniques to prolong the window of fertility for women has the ability to meet the needs of future populations and their delay in childbearing. If a viable source of oocyte production remains in infertile women with a reduced ovarian follicle

pool, for example, due to chemotherapy or advanced age, the potential exists to restore fertility in these women. The identification of GSCs gives hope to these women and suggests the potential for fertility restoration. In future, the protocols for isolation and culture of GSCs must be optimized. In the meantime, production of germ cells from ESCs or iPSCs is another possible alternative for the treatment of infertility.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Singleton Pregnancy Outcomes after In Vitro Fertilization with Fresh or Frozen-Thawed Embryo Transfer and Incidence of Placenta Praevia

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The aim of the study was to compare the single pregnancy and neonate outcome after fresh and frozen-thawed embryo transfer in the in vitro fertilization programme (IVF). The study focused on clinical and laboratory factors affecting the abnormal placentation, especially placenta praevia, in patients conceiving in the IVF programme. The results confirm that neonates born after frozen-thawed embryo transfer had significantly higher mean birth weight than after fresh embryo transfer (ET). Moreover, the birth weight distribution in singletons was found to shift towards “large for gestation” (LGA) after frozen-thawed ET. On the other hand, the pregnancies after fresh ET were characterized by a higher incidence of placenta praevia and 3rd trimester bleeding. Placenta praevia was more common in IVF patients with fresh ET in a stimulated cycle than in patients with ET in a spontaneous cycle. It occurred more frequently in patients with transfer of 2 embryos. From this point of view, single ET and ET in a spontaneous cycle should be encouraged in good prognosis patients in the future with more than two good quality embryos developed. An important issue arose of how the ovarian hormonal stimulation relates to abnormal placentation and if the serum hormone levels interfere with in the IVF treatment results.

1. Introduction

The reduction in number of embryos transferred into the uterus is crucial in preventing multiple pregnancies, one of the major in vitro fertilization (IVF) complications, since it is known that they carry increased risk for maternal and neonatal morbidity in comparison to singleton pregnancies [1–3]. Frozen-thawed embryo transfer (ET) technique enabled a transfer of fewer embryos into the uterus and storage of surplus embryos by cryopreservation for future use [3, 4]. The consequence of such “preventing multiplicity” practice is a rapidly increasing number of frozen embryos, followed by an increased percentage of births and neonates following such infertility treatment [2–5]. It is vital to confirm that newborns born after frozen-thawed ET are at least as healthy as the newborns born from fresh ET procedures [3, 5–12].

Until now no significant differences regarding children's outcome have been established in comparison to fresh ET

since the worldwide introduction of frozen-thawed ET into the everyday clinical practice [5–8, 10, 11, 13]. The studies have shown that neonatal outcome after frozen-thawed ET is comparable to the outcome after fresh ET [3, 5–11, 13]. Even though there were no major differences found in the outcome, it was nevertheless indicated that neonates born after frozen-thawed ET seem to have a higher birth weight. Furthermore, there was a decline in placenta praevia and antepartum haemorrhage after frozen-thawed ET in comparison to fresh ET [5, 8–13].

Placenta praevia and certain other placental abnormalities, followed by antepartum haemorrhage, generally occur more often in the IVF pregnancies and the causes are still poorly understood [2, 9, 12, 14, 15]. It has been speculated that placenta praevia more often develops after an IVF treatment because embryos are transferred via vagina and cervix [14]; however, a more recent study on gamete intrafallopian transfer (GIFT) showed a similar rate of placenta praevia

and we can therefore establish that the association between placenta praevia and ET procedure is less probable [12]. Other placentation abnormalities with an increased incidence in IVF pregnancies are placenta accreta [16, 17], vasa praevia [18, 19], and abnormal umbilical cord insertion [20]. Some of the crucial IVF pregnancy complications, such as intrauterine growth restriction (IUGR), low birth weight, and premature delivery [2, 3, 10, 21–23], could, to some extent, be the consequences of the above-mentioned placental abnormalities [2, 14, 17].

The aim of the study was to evaluate the differences between the singleton pregnancy outcomes after fresh or frozen-thawed ET in the IVF programme. The additional aim was to identify possible connections of placenta praevia to clinical parameters related to IVF procedure (including stimulation protocol, number of oocytes, number of developed and transferred embryos, and embryo quality), since the phenomenon of increased placenta praevia rates in IVF-derived pregnancies is still poorly understood.

2. Material and Methods

We analyzed the differences between pregnancy outcomes after fresh and frozen-thawed ET in our population of patients. Additionally, we analyzed the connections of placenta praevia to clinical parameters in women who conceived after in vitro fertilization procedure at our department.

2.1. Patients. In this retrospective cohort study we analyzed the outcomes of 1126 singleton pregnancies after IVF-ET, 211 of which have resulted from frozen-thawed ET and 915 pregnancies from fresh ET. The IVF-ET procedures were performed at the Department of Human Reproduction, University Medical Centre Ljubljana, in the time period between January 2004 and December 2011.

2.2. Ovarian Stimulation. Short antagonist cetorelix protocol (Cetrotide: Serono, London, UK, or Asta Medica AG, Frankfurt, Germany) or long desensitization buserelin protocol (Suprefact; Hoechst AG, Frankfurt/Main, Germany) was used for ovarian stimulation. The short protocol consisted of GnRH antagonist cetorelix acetate in a dose of 0.25 mg being administered at the time; the dominant follicle measured ≥ 14 mm in diameter after daily stimulation with 225 IU of follicle stimulating hormone (Gonal-F: Serono, Switzerland, Puregon) from day 2 of the menstrual cycle. In the long protocol buserelin was used until the criteria for ovarian desensitization were fulfilled (estradiol < 0.05 nmol/L, follicles < 5 mm in diameter) from day 22 of the menstrual cycle at a daily dose of 0.6 mL (600 pg) subcutaneously. Afterwards, 225 IU of follicle stimulating hormone was administered daily. All patients were administered 10,000 IU of human chorionic gonadotrophin (hCG) (Pregnyl; N.V. Organon) when at least three follicles were measured ≥ 17 mm in diameter.

2.3. Oocyte Retrieval, Fertilization, and Embryo Culture and Transfer. Transvaginal ultrasound-guided oocyte retrieval was performed 34 hours after hCG administration and under

local anesthesia. Fertilization of oocytes was performed using classical in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI).

Embryos were cultured to the blastocyst stage in sequential media M1 and M2 (Origio/MediCult, Jyllinge, Denmark) until day 5. One or two best quality blastocysts were transferred in the uterus on day 5. The blastocyst grading system introduced by Gardner et al. [24] was used to evaluate the blastocyst quality. Supernumerary blastocysts were frozen on day 5 or day 6 using our original modified two-step Menezo method described by Virant-Klun et al. [25] and stored in liquid nitrogen at -196°C . We rarely performed day 2 or day 3 ET of cleavage stage embryos in those patients with previous blastocyst development failure and only one or two embryos developed.

When needed, the cryopreserved blastocysts were thawed on the day of the transfer. Coordination between the endometrium and blastocysts in the frozen-thawed cycles was performed as described in Virant-Klun et al. [25]. One or two survived blastocysts with more than 50% of nondamaged cells were transferred 4 days after the disappearance of the dominant follicle in the natural cycle in women with regular menstrual patterns. In women with irregular menstrual cycles a minimal stimulation with 75 IU of rFSH was started daily from day 7 onward and the ET of one or two survived blastocysts was performed 6 days after the induction of ovulation with 5000 IU of hCG.

Luteal phase support consisted of 5000 IU of hCG on the day of ET in women receiving ovarian stimulation. Alternatively, 3×200 mg daily doses of progesterone were given intravaginally (Utrogestan: Viatrix Pharma, Paris, France) until a gestational sac with fetal heart activity was visible on day 30 after the ET procedure. Pregnancies (PR) were diagnosed by beta hCG serum determinations 14 days after hCG administration and by a vaginal ultrasound examination 4 weeks after the ET. Clinical PR was defined by an ultrasound observation of a positive heartbeat on day 30 after the ET procedure.

2.4. Data Collection. Data on the IVF procedures were collected in the IVF laboratory at our department on a daily basis. All 14 Slovenian maternity hospitals systematically collect data on maternal demographic characteristics, medical, gynecological and reproductive history, prenatal care, pregnancy, delivery, postpartum period, and neonates for each mother-infant pair using the same definitions of variables and the same form of medical record. The data are sent to the National Institute of Public Health of the Republic of Slovenia by default and were available for this research.

The data obtained from NPIS (National Perinatal Information System) were depersonalized in order to ensure the anonymity of the women and neonates. The research was performed according to the Personal Data Protection Act (UL). The research was approved by the National Medical Ethics Committee.

Slovene reference standard curves for weight, length, and head circumference at birth for given gestational age were used for fetal and neonatal growth estimation [26]. LGA

TABLE 1: Clinical characteristics of patients having fresh and frozen-thawed ET.

	Frozen-thawed ET Number of cases (%)	Fresh ET Number of cases (%)	P value
Number of patients	211	915	
Mean age (years \pm SD)	33.5 \pm 3.7	33.4 \pm 4.0	0.724
Multiparous	80 (39.7)	232 (25.4)	* <0.001
Previous abortions (mean, range)	0.46 (0–4)	0.33 (0–6)	* 0.011
Single ET	51 (24.2)	164 (17.9)	* 0.041
Double ET	160 (75.8)	751 (82.1)	
Blastocyst transfer	194 (91.9)	802 (87.5)	
Double blastocyst transfer	142 (67.3)	454 (49.6)	
Previous IVF attempts (mean \pm SD)	1.89 \pm 1.2	2.19 \pm 2.1	
1 attempt	117 (55.5)	406 (44.5)	
2 attempts	46 (21.8)	248 (27.2)	
3 attempts	32 (15.2)	112 (12.3)	* 0.03
4 attempts	9 (4.3)	73 (8.0)	
5 attempts	2 (0.9)	29 (3.2)	
6 attempts	3 (1.4)	15 (1.6)	
7 attempts	2 (0.9)	11 (1.2)	
8 and more attempts	0	19 (2.2)	
Infertility cause			
Tubal	52 (24.6)	253 (27.7)	0.511
Endometriosis	43 (20.3)	187 (20.4)	0.982
Endocrine	44 (20.9)	198 (21.6)	0.931
Male	39 (18.5)	166 (18.1)	
Others**	33 (15.6)	111 (12.1)	0.277

*Statistically significant ($P < 0.05$), as revealed by Chi-square or Mann-Whitney test. **Other causes include oncologic patients and idiopathic infertility.

(“large for gestational age”) was defined as growth above the 95th percentile and SGA (“small for gestational age”) was defined as growth below the 5th percentile according to the aforementioned population growth curves.

Preeclampsia was defined as arterial hypertension of at least 140/90 mm Hg detected for the first time in a pregnancy of at least 20 weeks or higher (measured at least 2 times and at least 6 hours apart). Additionally, at least 300 mg of proteins in daily urine collection must be present.

First trimester bleeding was defined as bleeding in the first 12 weeks of pregnancy, measured from the first day of the last menstrual bleeding (or calculated as 10 weeks after oocyte collection in women without menstruation), second trimester bleeding was defined as bleeding from 13 to 24 weeks of pregnancy, and third trimester bleeding was the one occurring after fulfilled 25 weeks of pregnancy.

Placenta praevia was diagnosed in cases of placental edge lying closer than 2 cm to the internal cervical os or over it. Placenta accreta was reported to the NPIS by the obstetrician who was performing a manual placenta removal, a postpartum abrasion, a caesarian section, or sometimes a removal of placental polyp. The report is written directly after the surgical procedure by the surgeon involved in the procedure.

2.5. Statistics. Statistical analysis was performed using IBM SPSS Statistics, version 19 (IBM Corporation, Armonk, NY). Student's *t*-test was used to compare normally distributed parametrical variables, Mann-Whitney's test was used to compare non-normally distributed variables, and Chi-square test was used to compare categorical variables. A multivariate logistic regression was used to identify independent risk factors. *P* value of < 0.05 was considered significant.

3. Results

The patients' mean age was 33.4 ± 3.9 years. Among these patients, 653 (58%) women were treated because of female factor infertility, 202 (18%) women because of male factor infertility, and 271 (24%) women because of both female and male factor infertility.

3.1. Comparison of Pregnancy Outcomes after Fresh and Frozen-Thawed ET

3.1.1. General. The patients' mean age, parity, previous abortions, number of embryos transferred, number of blastocyst transfers, and number of previous IVF attempts are presented in Table 1.

TABLE 2: Comparison of birth characteristics and neonate outcome following frozen-thawed and fresh ET.

	Frozen-thawed ET N = 211	Fresh ET N = 915	P value
Birth weight (g \pm SD)	3354.9 \pm 626.3	3200.1 \pm 632.7	*0.001
Mean gestation age (weeks \pm SD)	38.6 \pm 2.59	38.5 \pm 2.51	0.558
Preterm birth** (%)	9.0	12.1	0.232
Foetal distress (%)	3.3	3.7	1.000
Caesarean section (%)	25.1	25.8	0.930
Admittance to neonatal intensive care unit (%)	5.7	7.4	0.458
Low (0–6) 5-minute Apgar score (%)	1.4	1.6	1.000
Congenital malformation (%)	6.2	7.4	0.657
Chromosomal malformation (%)	0	0.1	1.000

*Statistically significant ($P < 0.05$), as revealed by t -test. **Preterm birth—birth before 37 weeks of gestation.

TABLE 3: Comparison of placental and amniotic abnormalities between pregnancies after frozen-thawed and fresh ET.

	Frozen-thawed ET N = 211 Number of cases (%)	Fresh ET N = 915 Number of cases (%)	P value
Preeclampsia	2 (0.9)	28 (3.1)	0.098
1st trimester bleeding	14 (6.6)	74 (8.1)	0.570
2nd trimester bleeding	2 (0.9)	27 (2.9)	0.144
3rd trimester bleeding	0 (0.0)	18 (2.0)	0.034*
Polihydramnion	2 (0.9)	7 (0.8)	0.679
Oligohydramnion	3 (1.4)	18 (2.0)	0.781
Placenta accreta	7 (3.3)	29 (3.2)	0.831
Placenta praevia	0 (0.0)	32 (3.5)	0.002*
Retained placenta	10 (4.7)	34 (3.7)	0.553
Manual removal of placenta	11 (5.2)	51 (5.6)	1.000

*Statistically significant ($P < 0.05$), as revealed by Chi-square.

The mothers in the frozen-thawed ET group were significantly more often multiparous with significantly more abortions in the history than the mothers in the fresh ET group. They also had more blastocyst transfers and less previous IVF attempts.

3.1.2. Birth Characteristics and Neonate Outcome. The mean birth weight of neonates, born after frozen-thawed ET, was significantly higher than the birth weight in the fresh ET group, whereas other birth characteristics and neonate outcomes were similar in both groups. The results are shown in Table 2.

Birth weight of neonates born after frozen-thawed ET was significantly higher than the birth weight of neonates born after fresh ET. Since the difference in parity between the groups could have an influence on the difference in birth weight, we performed a multivariate analysis of parity and gestational weight. The birth weight showed to be an independent variable after the implementation of logistic regression and remained significantly higher in singletons after frozen-thawed ET ($P = 0.002$).

Analysis of birth weight distributions in both groups of neonates revealed a shift of singletons after frozen-thawed ET towards the LGA. There were 10.5% of neonates after the frozen-thawed ET and 5.0% of neonates after the fresh ET who were above the 95th centile of weight for a certain gestation the difference being significant ($P = 0.003$). In addition, there were significantly less SGA pregnancies in the frozen-thawed ET in comparison to the fresh ET group (0.9% versus 3.7%, resp., $P = 0.048$).

3.1.3. Abnormal Placenta and Complications during Pregnancy. Pregnancies after fresh ET were characterized by significantly higher rates of placenta praevia and 3rd trimester bleeding, as can be seen in Table 3. After frozen-thawed ET we did not find any cases of placenta praevia and 3rd trimester bleeding.

Placenta praevia was the only pathology that differed significantly between the two groups.

We analyzed the occurrence of placenta praevia in primiparous and multiparous patients in both ET groups to establish the influence of parity on the rate of placenta praevia

TABLE 4: IVF-ET cycle characteristics resulting in pregnancies with and without placenta praevia.

	Pregnancies with placenta praevia N = 32	Pregnancies without placenta praevia N = 1095	P
Day of ET			
2	0	14 (1.3%)	0.775
3	0	9 (0.8%)	
4	0	1 (<0.1%)	
5	32 (100%)	1037 (94.7%)	
6	0	34 (3.1%)	
Number of morulae and blastocysts on day 5			
2 or 3	18 (56%)	610 (56%)	0.410
4 or 5	10 (31%)	258 (23%)	
6 or more	3 (3%)	222 (21%)	
ET number (1 or 2) and embryo quality (poor/good)**		*	
1—poor embryo	0	13 (1%)	0.491
2—poor/poor embryo	3 (9%)	76 (7%)	
1—good embryo	6 (19%)	153 (14%)	
2—good/poor embryo	2 (6%)	197 (18%)	
2—good/good embryo	21 (66%)	461 (42%)	
Stimulation protocol		*	
Long protocol	18 (56%)	685 (63%)	0.403
Short protocol	14 (44%)	359 (33%)	

*24 data on stimulation in a group of pregnancies without placenta praevia missing and 18 data on embryo quality in group without placenta praevia missing.

**Good quality: good blastocysts quality according to Gardner criteria [24] on day 5 or 6, cleavage stage embryo with little (<10%) or no fragmentation on day 2 or 3, and equal (round) blastomeres on day 2 or 3. Poor quality: embryos not reaching the criteria for good quality.

cases. There were significantly less pregnancies with placenta praevia in the group of pregnancies resulting from frozen-thawed ET in multiparous women (4.3% versus 0%; $P = 0.049$) as well as in primiparous women (3.2% versus 0%; $P = 0.020$).

3.2. Parameters Related to Abnormal Placentation regardless of Embryo Transfer Type. We performed additional analysis of all pregnancies with placenta praevia in order to establish possible background factors that could be connected to pathogenesis. There were no significant differences in the number of oocytes retrieved, oocytes fertilized, and day of ET or embryo quality between IVF cycles of patients with and without placenta praevia (see Table 4).

Mean number of oocytes retrieved in fresh cycles with and without placenta praevia was similar (9.7 ± 5.5 oocytes versus 9.2 ± 5.2 oocytes, resp., $P = 0.830$).

To perform a multivariate analysis of abnormal placentation we included all cases of placenta praevia, accreta, and retained placenta that together formed a larger group of 99 pregnancies. Using logistic regression we examined connection between background parameters (maternal age, parity, uterine surgery, supernumerary embryos, number of embryos transferred, and body mass index (BMI)) and abnormal placentation.

There was no connection of abnormal placentation to maternal age, parity, uterine surgery, supernumerary

embryos, or BMI. Transfer of 2 embryos was a significant factor for occurrence of abnormal placentation and spontaneous cycle ET (i.e., ET without any stimulation) was of borderline significance (P value being between 0.050 and 0.100) for absence of abnormal placentation. Results are shown in Table 5.

The process of comparing the groups of women with and without abnormal placentation showed no difference in terms of previous caesarean sections (CS), the type of the fertilization procedure (IVF, ICSI/TESE), or number of retrieved oocytes in the IVF cycle. The results are shown in Table 6.

4. Discussion

The results of this study showed that the singletons after frozen-thawed ET had significantly higher mean birth weight than the singletons after fresh ET. Moreover, the birth weight distribution in singletons after frozen-thawed ET was found to shift towards LGA. On the other hand, the pregnancies following fresh ET were characterized by a higher incidence of placenta praevia and 3rd trimester bleedings than the pregnancies after frozen-thawed ET. The abnormal placentation rates were unrelated to most IVF-associated parameters, apart from the double blastocyst transfer and transfer in a spontaneous cycle.

We performed multivariate analysis in order to establish whether the disproportion in parity influenced the difference

TABLE 5: Multivariate analysis using logistic regression showing connection of abnormal placentation to background parameters in 99 women.

Parameter	<i>P</i>	Odds ratio (OR)	95% confidence interval (CI)
Maternal age	0.846	0.995	0.943–1.050
Multiparity	0.293	0.751	0.459–1.235
Previous uterine surgery (including myomectomy, caesarean section, reconstructive surgery, or septum resection)	0.490	1.132	0.718–1.784
BMI			
Extremely low (under 18.5)	0.402	0.538	0.126–2.290
High (above 30)	0.544	0.764	0.320–1.823
Supernumerary embryos developed in IVF cycle	0.275	1.269	0.827–1.946
Transfer of 2 embryos	0.043	1.952	0.020–3.735
Spontaneous cycle ET	0.064	0.417	0.150–1.164

TABLE 6: Previous caesarean sections, number of oocytes retrieved, and fertilization procedure in women with and without abnormal placenta.

Parameter	Pregnancies with abnormal placenta <i>N</i> = 99 <i>N</i> (%)	Pregnancies without abnormal placenta <i>N</i> = 1028 <i>N</i> (%)	<i>P</i>
Previous CS	4 (4)	31 (3.1)	0.538
Number of oocytes retrieved (mean \pm SD)	10.0 \pm 5.7	10.0 \pm 5.6	0.687
IVF	54 (54.6)	591 (57.5)	
ICSI/TESE	44 (44.4)	417 (40.6)	
	1 (1.0)	20 (1.9)	0.671

in birth weights between the frozen-thawed and fresh ET neonates. The birth weight of frozen-thawed ET neonates remained significantly higher than the birth weight of fresh ET neonates, which is consistent with the results found in the literature [5, 7–10, 13, 22, 27]. Birth weight of frozen-thawed ET neonates proved to be a parity-independent variable with a significant shift towards LGA, which has also been discovered by other studies [9, 13, 22, 27]. The delivery of a LGA neonate represents a greater morbidity risk for the mother and neonate [28], yet another concern is the possible hidden epigenetic pathology. It is known that some rare epigenetic disorders occur more often after ART than after spontaneous conception, such as Beckwith-Wiedemann syndrome, Angelman syndrome, and possibly cancer [29, 30]. High incidence of “large offspring syndrome” has been noticed following ART of cattle and sheep resulting in high birth weight which has recently been proven to be epigenetically and phenotypically similar to Beckwith-Wiedemann’s syndrome in humans [29, 30]. However, no such pathology has been observed in our population, nor in the whole Danish population [31]; therefore, other causes should also be considered [9, 13, 29]. The SGA and LBW rates were significantly reduced in the frozen-thawed ET group, thus providing us with similar results as observed by other authors [8–10, 13, 22].

The study revealed significant differences regarding abnormal placentation that more often occurs in pregnancies following ART and mostly for unknown reasons [2, 3, 9, 12, 14, 19, 32–34]. It was established that IVF conception

substantially increases the risk for early preeclampsia cases [32], which is often explained by primiparity of the IVF patients. The rates of preeclampsia in our study were three times lower in the frozen-thawed ET group ($P = 0.098$) than in the fresh ET group, which is the opposite of the results obtained in a national Swedish study [9]. Our findings can be explained by a higher rate of multiparous mothers in frozen-thawed ET group, while the Swedish results remained unclarified [9].

Similarly, placenta praevia, accreta, and vasa praevia more often occur in pregnancies derived from an IVF procedure [2, 12, 14, 17, 19] and with unfavorable consequences [15]. The placenta praevia rates in our and other reports are higher in multiparous women [15] but lower in the frozen-thawed ET group [9, 12]. Since in frozen-thawed ET group there were significantly more multiparous women, but significantly lower placenta praevia rates, parity could not in any case lower the placenta praevia rate in this group of pregnancies. On the other hand, there was no difference in placenta accreta, manually removed placenta, and retained placenta cases between the frozen-thawed and fresh ET groups in our study.

Australian report discovered less haemorrhages in pregnancies after frozen-thawed and spontaneous cycle ET [12]. Israeli investigators found the connection between abnormal placentation and higher estradiol serum levels [17]. Interestingly, there were no variations between oocytes retrieved in cycles with and without placenta praevia in our study that would indicate high ovarian response being the risk factor

for placenta praevia in general. Our study was larger than the Israeli study (32 cases of placenta praevia versus 2 cases, resp.); however, we did not include the serum estradiol measurements into the analysis. Since we did not find any cases of placenta praevia in pregnancies after ET in a spontaneous cycle, we can conclude that the hormonal stimulation might have influenced the manifestation of placenta praevia. With reference to our and other studies [10, 12, 17, 22], the effect of hormonal stimulation, including the estradiol levels at the time of implantation, needs to be investigated further.

The manifestation of placenta praevia was significantly related to the transfer of two embryos, all of them followed day 5 ET. However, there were 95% transfers of blastocysts in this period in our IVF programme, since we adopted the blastocyst transfer policy early. Therefore the impact of blastocyst transfer versus cleavage stage embryos on the pregnancy outcome is difficult to evaluate in this patient population. The Swedish analysis has associated placenta praevia with the blastocyst transfer [35]. Contrastingly, a recent large Japanese study (over 270.000 single ET) established that the blastocyst transfer was not associated with placental abnormalities, including placenta praevia; however, frozen-thawed ET was indeed associated with the risk of placenta accreta. [27]. In a recent meta-analysis, which did not include the study mentioned above, blastocyst transfer was linked to higher risk of preterm birth [11], while the Japanese study revealed reduced low birth weight rates and increased LGA weight after transferring blastocysts [27].

In contrast to expected risk factors for placenta praevia observed in the general population, previous caesarean section and uterine surgery were not connected to abnormal placentation in our study population. Similarly, higher parity and abortion rates were not risk factors for placenta praevia in pregnancies following frozen-thawed ET.

Our current study points to the fact that abnormal placentation more commonly occurs in IVF patients with fresh ET in a stimulated cycle and it is more common among patients with the transfer of 2 embryos. From this point of view, single ET and ET in a spontaneous cycle should be encouraged. It seems that the reasons for placenta praevia in the IVF programme differ from those found in the general population, which are caesarean sections, multiparity, or uterine surgery; all these factors did not significantly influence the placentation in our IVF population of patients.

5. Conclusion

The results of our study revealed significantly more LGA neonates in pregnancies after frozen-thawed ET in comparison to fresh ET outcome. Does the higher birth weight indicate a more healthy pregnancy, or is this birth weight shift pathological, carrying all the risks of LGA, metabolic disorders, or even epigenetic changes? Placenta praevia seems to be a complication related to stimulated cycles with 2 fresh embryos transferred. The known risk factors for placenta praevia in the general population were not significant in our study population and we may therefore conclude that the patients conceiving in the IVF programme represent a special

population. Such investigations give us the opportunity to research the possible background of still poorly understood abnormal placentation. Better results after frozen-thawed ET and ET in a spontaneous cycle imply that the environment at the moment of ET is important and may influence the pregnancy outcome. How does the hormonal stimulation relate to these pathologies? It is not excluded that IVF pregnancies possess somehow altered mechanisms of placentation, foetal development, and growth.

Conflict of Interests

The authors declare that there is no financial or other conflict of interests related to this paper.

Authors' Contribution

All authors contributed to the paper.

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Research Article

Clinical and Structural Features of Sperm Head Vacuoles in Men Included in the In Vitro Fertilization Programme

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The human sperm head vacuoles and their role in male infertility are still poorly understood. The aim of this study was to identify the clinical and ultrastructural features of human sperm head vacuoles in men included in the in vitro fertilization programme: men with normal (normozoospermia) and impaired sperm morphology (teratozoospermia). The sperm samples were observed under 6000-time magnification using motile sperm organelle morphology examination (MSOME). The proportion of sperm with head vacuoles was evaluated and related to the outcome of in vitro fertilization. The sperm of men with impaired sperm morphology was characterized by a higher proportion of sperm head vacuoles. The sperm head vacuoles were related to impaired semen quality (sperm concentration, motility, and morphology) but were not influenced by male factors (semen volume, height, age, weight, or body mass index). Moreover, sperm head vacuoles were related to impaired fertilization rate merely after classical in vitro fertilization (IVF), while there was no relation to pregnancy. In a subgroup of men, the sperm was fixed and observed by transmission electron microscopy (TEM). The ultrastructural study revealed that sperm head vacuoles are large nuclear indentations of various sizes and positions, packed with membranous material organized in membrane whorls (MW).

1. Introduction

Male infertility can be related to abnormal morphology of spermatozoa. In the last years, there has been much debate on a new potential parameter of sperm quality and male fertility, sperm head vacuoles. In spite of that, the human sperm head vacuoles and their potential role in male infertility are still poorly understood.

Using motile sperm organelle morphology examination (MSOME), where living sperm cells are visualized with DIC Nomarski microscopy at magnification of 6000 times, some fine morphological structures are visible which might be useful to determine the quality of sperm. This method shows small and larger surface concavities termed sperm vacuoles, usually located in the head of sperm cells. The nature and origin of sperm head vacuoles are still uncertain and it is not

clear whether they originate from the nucleus or acrosome or other membranous structures. Vacuoles are also found in almost all samples with normal sperm morphology (>14% morphologically normal spermatozoa according to Kruger's strict criteria) and are mainly located at the tip or middle area of sperm heads [1]. The three-dimensional deconvolution images, which detect DNA, showed vacuoles as "nuclear concavities" and atomic force microscopy revealed intact but slightly sunken plasma membrane in the area of vacuoles [2]. Moreover, the electron microscope images of sperm samples in patients with abnormal sperm morphology, teratozoospermia (<14% normal spermatozoa in the sample according to Kruger's strict criteria), showed vacuoles exclusively in the area of sperm nucleus [3]. The anterior location of sperm head vacuoles led to the question about their possible origin from acrosome. The induction of acrosome reaction by

ionophore A23587 [4], follicular fluid, and hyaluronic acid [5] decreased the percentage of spermatozoa with head vacuoles which suggested that they might be related to the acrosome. However, vacuoles are also present in the sperm samples without acrosome (i.e., globozoospermia), suggesting that they do not originate from acrosome [6]. It was shown that samples with large sperm head vacuoles (more than 13% of head surface) have higher percentage of fragmented DNA compared to those with small vacuoles [7] as well as higher frequency of chromosome abnormalities [3]. The sperm head vacuoles observed under the high magnification of light microscopy were described as “pocket-like” nuclear concavities related to failure of chromatin condensation [3, 8]. On the contrary, some researchers did not find any correlation between the large sperm head vacuoles and DNA damage [1] or chromosome abnormalities [1, 2]. The sperm nucleus modelling during spermiogenesis involves nucleoproteins, microtubular manchette, and perinuclear material which is known to participate in the acrosome anchorage on the nucleus and also constitutes the perinuclear theca [9]. During spermiogenesis, the nucleus becomes strikingly reduced and the nucleosomes usually appear nonrandomly distributed throughout the genome. The nucleosome-bound DNA may stay assembled with nuclear matrix and is seen as “empty space” in electronic images. Together they could have important role in paternal chromatin structure following fertilization [10]. In the late steps of spermiogenesis, the nuclear DNA becomes highly condensed because the nucleoproteins histones are replaced by protamines [11]. During replacement of nucleoproteins, many temporarily functional proteins are formed and degraded in special degradation sites. The nuclear pockets at the base of sperm head were proposed to function as proteolytic centers and the exit site from where the protein residues leave the nucleus and may be functionally connected with vacuoles [11, 12].

The sperm head vacuoles are an interesting but poorly understood parameter of sperm quality and male fertility which seem to be related to the outcome of in vitro fertilization in men with impaired sperm quality [13–15]. The origin and dynamics of sperm head vacuoles are not well understood and their role in sperm function is disputable. Some authors suggest that sperm vacuoles are normal features of sperm head [1, 16], while others describe them as degenerative structures related to male subfertility [2, 17, 18]. Therefore, the aim of this study was to evaluate the percentages of sperm with head vacuoles in both the normal sperm samples and samples with impaired sperm morphology and to relate them to the outcome of in vitro fertilization. Moreover, the ultrastructure and function of sperm head vacuoles were reconsidered in relation to sperm maturation, using the high resolution of transmission electron microscopy.

2. Materials and Methods

In this study, the sperm samples of 81 men included into the in vitro fertilization programme were examined: 40 men with normal sperm morphology and 41 men with abnormal sperm morphology (teratozoospermia) according to Kruger's

strict criteria (abnormal: <14% normal sperm). The sperm samples were prepared and used for in vitro fertilization. Each sperm sample was prepared by centrifugation (300 g) for 20 min on 100%/40% density gradient of PureSperm (NidaCon, Sweden) followed by the swim-up technique, where the best spermatozoa were extracted from the sample. All normal sperm samples were used for classical in vitro fertilization (IVF), while the samples with abnormal sperm morphology were used for intracytoplasmic sperm injection (ICSI) to fertilize the female partner's oocytes. The samples, which remained after in vitro fertilization, were used for motile sperm organelle morphology examination (MSOME) to evaluate the percentage of sperm head vacuoles in each sample under 6000-time magnification. The percentages of sperm with head vacuoles and the outcome of in vitro fertilization were compared between the normal and abnormal sperm samples. Using the high resolution of transmission electron microscopy (TEM), the ultrastructure of 10 samples of normozoospermic men and 6 samples of teratozoospermic men was observed. The potential function and structure of vacuoles were searched, also in relation to sperm maturation process.

This research was approved by the National Medical Ethics Committee.

2.1. Classical Parameters of Semen Quality. The classical parameters, sperm concentration and motility and morphology of each sperm sample, were evaluated. The sperm sample was supposed to be normal at concentration ≥ 15 million spermatozoa per mL and $\geq 40\%$ total (progressive and non-progressive) motile spermatozoa according to WHO criteria [19] and at $\geq 14\%$ of morphologically normal spermatozoa according to Kruger's strict criteria.

2.2. Motile Sperm Organelle Morphology Examination (MSOME). For motile sperm organelle morphology examination, we used droplets of SpermSlow medium (Origio, Denmark) in a glass-bottomed dish (GWSt1000; Will Co, Wells, Amsterdam, The Netherlands) and droplets of sperm sample prepared by swim-up technique, previously described by Knez [20]. The spermatozoa in each sample were monitored under an inverted microscope with a heated stage equipped with differential interference contrast (Eclipse TE2000-S; Nikon, Japan). One thin, elongated droplet of SpermSlow medium (Origio, Denmark) was placed on the bottom of a glass dish. A smaller droplet of prepared spermatozoa was placed near SpermSlow droplet and spermatozoa bound to hyaluronate. All droplets were covered with paraffin oil to prevent drying of the sperm sample. For observation under 6000 magnification, a droplet of immersion oil was inserted underneath the glass dish (under the SpermSlow droplet). A droplet of SpermSlow with bound spermatozoa was monitored by the immersion objective, differential interference contrast, and a Nikon Digital Sight DS-R1 camera. In each sample, 200 spermatozoa bound to the hyaluronate were chosen and evaluated according to head vacuoles, at 6000-time magnification. The scoring system for the evaluation of each

TABLE 1: Sperm quality in normospermic men and in men with teratozoospermia: classical parameters of sperm quality and sperm head vacuoles.

Sperm quality	Normozoospermia	Teratozoospermia
Number of patients	40	41
Mean concentration of sperm ($\times 10^6$ spz/mL)	95.5 (min. 50–max. 200)	48.4 (min. 2–max. 100)
Mean sperm motility (% of motile spz)	72.0% (min. 50–max. 80)	65.1% (min. 14–max. 80)
Mean sperm morphology (% of normal spz)	35.6% (min. 18–max. 67)	8.0% (min. 2–max. 13)
% of sperm with head vacuoles	74.6%*	90.2%*
% of Class I sperm (no vacuoles)	26.5%*	9.7%*
% of Class II sperm (at most two small vacuoles)	47.1%	44.6%
% of Class III sperm (more than two small vacuoles or one big vacuole)	25.4%**	37.8%**
% of Class IV sperm (big vacuoles)	2.1%*	7.8%*

*Statistically significant difference ($P < 0.01$).

**Statistically significant difference ($P < 0.05$), as revealed by chi-square test.

spermatozoon was based on the following classification of Vanderzwalmen [14]. According to the number of vacuoles and their size, spermatozoa were classified into the following: (1) Class I: absence of vacuoles, (2) Class II: maximum of two small vacuoles, (3) Class III: more than two small vacuoles or at least one large vacuole, and (4) Class IV: large vacuoles in conjunction with abnormal head shapes or other abnormalities. In each sperm sample, the proportions of Class I, Class II, Class III, and Class IV spermatozoa were evaluated. For statistics, the proportions of Class I and Class II spermatozoa were combined into one group, “good sperm,” and the proportions of Class III and Class IV sperm into one group, “bad sperm,” in 3 terms of sperm head vacuoles.

2.3. Transmission Electron Microscopy (TEM). To describe the ultrastructural characteristics of vacuoles in sperm nucleus, chosen sperm samples were centrifuged at 500 g for 10 minutes at room temperature. Supernatants were carefully removed and pellets were fixed in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1M PB (Sørensen’s phosphate buffer), pH 7.0 for 24 h at 4°C, postfixed with 1% OsO₄ in 0.1M PB for 1.5 h, and embedded in Agar 100 resin (Agar Scientific). Ultrathin sections were stained with 4% water solution of uranyl acetate and Reynolds lead citrate. Samples were examined with Philips CM 100 electron microscope (FEI, The Netherlands), operating at 80 kV; images were recorded with Bioscan 792 and Orius 200 camera (Gatan) using Digital Micrograph, Software (Gatan Inc., Washington, DC, USA) at magnifications from 1100 to 92000 times.

2.4. Statistical Analysis. The statistical significance of differences in the percentages of sperm with sperm head vacuoles and the outcome of in vitro fertilization (pregnancy rate) between the normal sperm samples and samples with abnormal morphology (teratozoospermia) were evaluated by chi-square test. The percentages of “good sperm” (Class I and Class II), “bad sperm” (Class III and Class IV), and Class I, Class II, Class III, and Class IV sperm in terms of sperm head vacuoles were correlated with classical parameters of semen quality (concentration, motility, and morphology), male factors (semen volume, leukocytospermia, age, height,

weight, and body mass index), and the outcome of in vitro fertilization (fertilization, blastocyst, and pregnancy rates) using Spearman’s rho correlation. For the outcome of in vitro fertilization, the data were evaluated for total in vitro fertilization cycles and separately for classical in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). The pregnancy dependence on female and male factors (including sperm head vacuoles) was evaluated by multivariate logistic regression. The statistical significance was set at $P < 0.05$.

3. Results and Discussion

3.1. Motile Sperm Organelle Morphology Examination (MSOME)

3.1.1. Sperm Head Vacuoles as Seen by Light Microscopy of MSOME. In both, the normal and abnormal sperm samples, the vacuoles were located in different parts of the head of spermatozoa. They were seen as surface concavities or lighter translucent areas of various sizes and numbers. In literature, the sperm head vacuoles are described as surface concavities, pocket-like nuclear concavities [8], or nuclear thumb-prints [2] under the light microscope. In our study, the MSOME, using Nomarski DIC microscopy at 6000-time magnification, showed sperm head vacuoles as surface concavities (Figure 1). However, we must take into consideration that Nomarski DIC microscopy results in a pseudo-3D image due to refraction of light passing through the specimen of different thickness and optical density. Therefore, it is possible that nuclear regions of different optical density are not just concavities but also irregular bulges at the cell surface.

3.1.2. Sperm Head Vacuoles, Classical Parameters of Sperm Quality, and Male Factors. Our data show that sperm head vacuoles were present in both the normal and abnormal sperm samples (Figure 1). Interestingly, there was a relatively high proportion (74.6%) of spermatozoa with sperm head vacuoles in the sperm of normal quality, thus indicating that the sperm head vacuoles are also found in normospermic men (Table 1). Although there was a higher proportion of

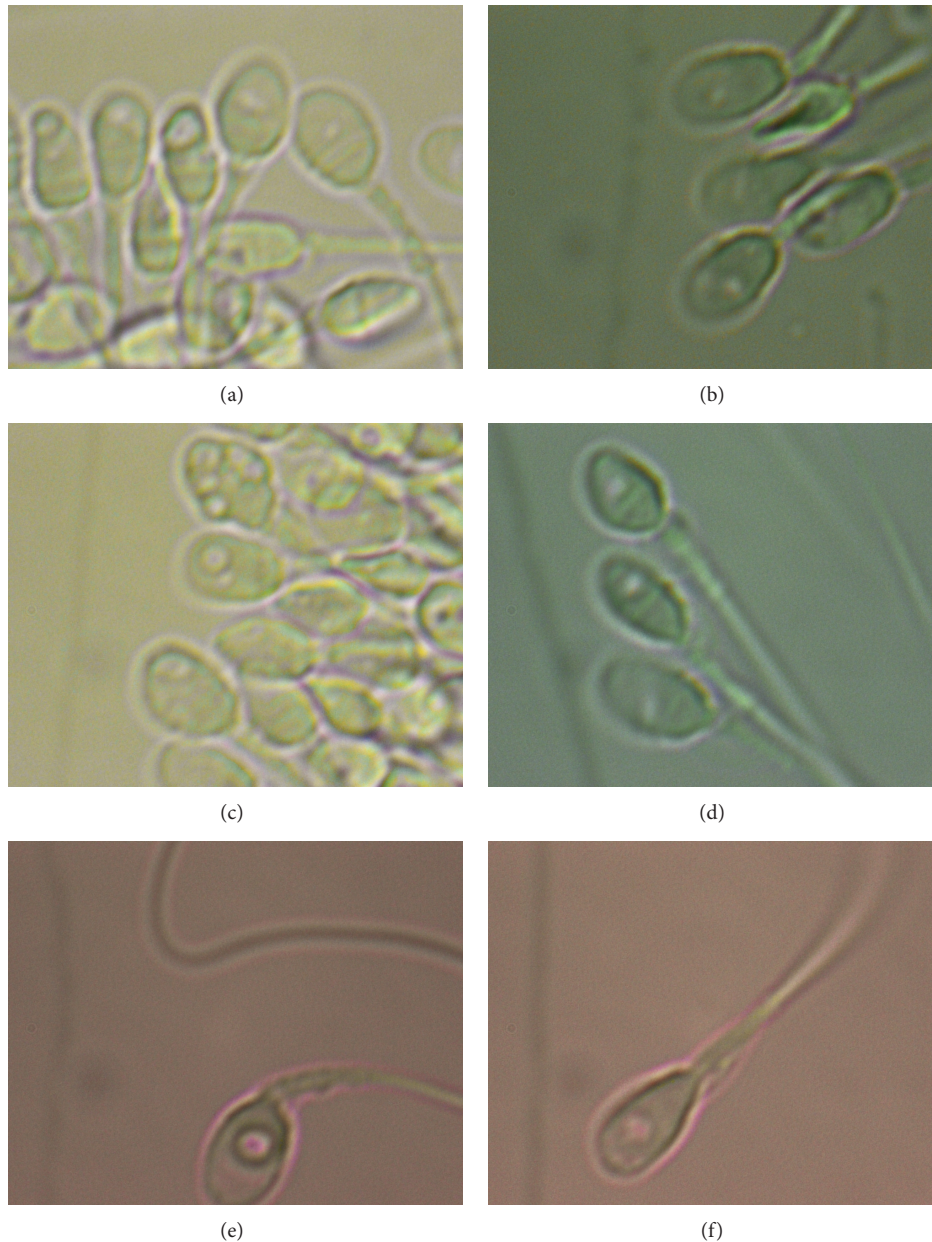


FIGURE 1: Sperm with head vacuoles in normozoospermic ((a), (c), and (e)) and teratozoospermic ((b), (d), and (f)) samples observed with DIC microscopy (Nomarski) at 6000-time magnification.

spermatozoa with head vacuoles in men with teratozoospermia than in men with normal sperm quality (90.2% versus 74.6%; $P < 0.01$), as can be seen in Table 1, it is not excluded that sperm head vacuoles may also be related to the sperm pathology in these men. Moreover, in the sperm of men with teratozoospermia, there were a significantly lower proportion of optimal spermatozoa of Class I and a higher proportion of low quality sperm of Classes III and IV than in samples of normal sperm (Table 1)

However, some previous studies reported higher percentages of vacuolated spermatozoa in subfertile men with

normal semen parameters, ranging from 97.7% [16] to 98.4% [21]. This discrepancy reflects the fact that the subfertile men with normal semen quality but included into the in vitro fertilization program represent a heterogeneous population of men, from fertile partners of infertile women to men with repeated poor in vitro fertilization outcome in spite of normal semen quality. In addition, there are several different habits, lifestyles, and health conditions (e.g., inflammation and varicocele) that may affect their sperm in terms of head vacuoles. There was a positive correlation between the proportion of “good sperm” (Class I and Class II) in

TABLE 2: Correlation between sperm head vacuoles (proportions of Classes I–IV) sperm with classical parameters of sperm quality (concentration, motility, and morphology).

	Correlations		
	Sperm quality		
	Concentration	Motility	Morphology
Spearman's rho			
I plus II			
Correlation coefficient	,233*	,201	,326**
Significance (2-tailed)	,037	,074	,004
N	80	80	78
III plus IV			
Correlation coefficient	–,238*	–,208	–,328**
Significance (2-tailed)	,033	,063	,003
N	80	80	78
I			
Correlation coefficient	,321**	,222*	,529**
Significance (2-tailed)	,004	,048	,000
N	80	80	78
II			
Correlation coefficient	,055	,111	–,091
Significance (2-tailed)	,627	,326	,429
N	80	80	78
III			
Correlation coefficient	–,188	–,138	–,275*
Significance (2-tailed)	,095	,222	,015
N	80	80	78
IV			
Correlation coefficient	–,217	–,225*	–,267*
Significance (2-tailed)	,054	,044	,018
N	80	80	78

*Correlation is significant at the 0.05 level (2-tailed).

**Correlation is significant at the 0.01 level (2-tailed).

terms of head vacuoles and sperm concentration and sperm morphology (percentage of normal sperm) in men included in this study, as can be seen in Table 2.

Moreover, there was a positive correlation between the proportion of Class I sperm and sperm concentration, motility, and morphology. On the other hand, there was a negative correlation between the proportion of “bad sperm” (Class III and Class IV) in terms of head vacuoles and sperm concentration and morphology (Table 2). In addition, the proportions of Class III and Class IV sperm were negatively correlated with sperm morphology and the proportions of Class IV sperm were also negatively correlated with sperm motility. All of these data indicate that sperm head vacuoles

were related to impaired sperm quality, although they were also present in normal sperm samples. This finding is in accordance with some previous studies [21].

Interestingly, there was a significant negative correlation between the male age and concentration of sperm, while we did not observe any correlation between the sperm head vacuoles and male factors such as age, height, weight, and body mass index (Table 3).

We also did not find any significant correlation between semen volume or leukocytospermia and sperm head vacuoles. On the other hand, there were more previous studies which showed that some of these male factors may have an impact on sperm quality and formation of head vacuoles [22–24]. One of this studies showed that increased male age and body mass index are related to increased sperm head vacuoles but this may be compensated by higher ejaculation frequency and a shorter period of sexual abstinence in these men [24]. This means that some other still unknown male factors affect the appearance of sperm head vacuoles.

3.1.3. Sperm Head Vacuoles and the Outcome of In Vitro Fertilization. The groups of men included into the study did not differ regarding the mean age of female partners and the number of oocytes per woman, the main female factors that may affect the outcome of in vitro fertilization (Table 4). The pregnancy rates achieved in teratozoospermic and normozoospermic men were 32.5% and 26.8% (see Table 4).

By further inspection, it became clear that the proportion of “good sperm” or “bad sperm” in terms of head vacuoles in semen samples was indicative for a proportion of fertilized oocytes (fertilization rate) after in vitro fertilization procedure but this depended on the method of in vitro fertilization. After classical in vitro fertilization (IVF), there was a significant positive correlation between the percentage of “good sperm” in the semen sample and fertilization rate (Table 5). On the other side, there was a negative correlation between the proportion of “bad sperm” and the fertilization rate, as can be seen in Table 5.

From Figure 2, it can be seen that, in semen samples with more than ~50% of “bad sperm” in terms of sperm head vacuoles, the fertilization rate was decreased. Interestingly, we did not observe any correlation between sperm head vacuoles and fertilization rate after intracytoplasmic injection (ICSI), as found by some previous studies [16]. This indicates that sperm head vacuoles may impair some physiological process of spermatozoa that are needed for classical in vitro fertilization by insemination such as capacitation, acrosome reaction, sperm binding to zona pellucida, or that some more sperm selection is performed by ICSI.

Regarding blastocyst rate, there was a negative correlation ($P < 0.05$) between the percentage of Class III spermatozoa in semen samples and blastocyst rate after classical in vitro fertilization, while we did not observe any other correlations. We did not observe any correlation between sperm head vacuoles and pregnancy rate regardless of the method of in vitro fertilization. There was a positive relation between the pregnancy and number of oocytes retrieved in female partner,

TABLE 3: Correlation between male factors (age, height, weight, and body mass index), classical parameters of sperm quality, and sperm head vacuoles.

	Correlations			
	Age	Height	Weight	BMI
Spearman's rho				
I plus II				
Correlation coefficient	-,183	-,009	-,066	-,098
Significance (2-tailed)	,124	,945	,595	,431
N	72	67	67	67
III plus IV				
Correlation coefficient	,178	,003	,060	,095
Significance (2-tailed)	,135	,978	,632	,446
N	72	67	67	67
I				
Correlation coefficient	-,150	,042	-,022	-,069
Significance (2-tailed)	,209	,733	,862	,578
N	72	67	67	67
II				
Correlation coefficient	-,038	-,085	-,071	-,054
Significance (2-tailed)	,748	,496	,570	,663
N	72	67	67	67
III				
Correlation coefficient	,137	,045	,086	,095
Significance (2-tailed)	,250	,716	,488	,442
N	72	67	67	67
IV				
Correlation coefficient	,036	-,042	-,073	-,037
Significance (2-tailed)	,762	,733	,558	,766
N	72	67	67	67
Concentration				
Correlation coefficient	-,246*	-,151	-,100	-,031
Significance (2-tailed)	,039	,225	,425	,807
N	71	66	66	66
Motility				
Correlation coefficient	-,138	-,116	,017	,078
Significance (2-tailed)	,250	,355	,890	,535
N	71	66	66	66
Morphology				
Correlation coefficient	-,151	-,154	-,108	-,027
Significance (2-tailed)	,215	,226	,397	,832
N	69	64	64	64

* Correlation is significant at the 0.05 level (2-tailed).

BMI: body mass index.

as revealed by Mann-Whitney *U* test, but we did not find any relation between pregnancy and sperm head vacuoles. This was further confirmed by multivariate logistic regression respecting female and male factors including sperm head vacuoles.

Most of the previous studies have tried to elucidate the effect of sperm head vacuoles on the outcome of ICSI in men with poor semen quality and some of them indeed confirmed

the negative effect [13–15]. The advantage of our study is that we elucidated the effect of sperm head vacuoles not only on the outcome of ICSI in semen samples of poor quality (teratozoospermia) but also on the outcome of classical IVF in normal semen samples (with normal classical parameters of semen quality, including morphology). Our data indicate that the sperm head vacuoles were even more deleterious for the outcome of classical IVF than for ICSI.

TABLE 4: In vitro fertilization outcome (pregnancy rate) according to the sperm quality (normozoospermia and teratozoospermia).

In vitro fertilization outcome	Normozoospermia	Teratozoospermia
Number of patients (cycles)	40	41
Mean number of previous cycles	2.0 (min. 1–max. 5)	1.2 (min. 1–max. 6)
Mean age of female partners (years)	34 (min. 25–max. 43)	34 (min. 25–max. 42)
Mean number of oocytes per woman (cycle)	7.7 (min. 1–max. 24)	8.0 (min. 1–max. 18)
Number of pregnancies	13	11
Pregnancy rate per cycle	32.5%	26.8%

Statistical significance was set at $P < 0.05$.

TABLE 5: Correlation between sperm head vacuoles (“good sperm”: Class I plus Class II; “bad sperm”: Class II plus Class IV) and outcome of in vitro fertilization (fertilization and blastocyst rates) according to the method of in vitro fertilization.

Correlations			
Method	I plus II	III plus IV	
IVF			
Fertilization rate			
Correlation coefficient	,358*	–,358*	
Significance (2-tailed)	,041	,041	
N	33	33	
Blastocyst rate			
Correlation coefficient	,261	–,261	
Significance (2-tailed)	,207	,207	
N	25	25	
ICSI			
Fertilization rate			
Correlation coefficient	–,050	,048	
Significance (2-tailed)	,738	,747	
N	48	48	
Blastocyst rate			
Correlation coefficient	–,041	,042	
Significance (2-tailed)	,809	,805	
N	37	37	

*Correlation is significant at the 0.05 level (2-tailed).
IVF: classical in vitro fertilization; ICSI: intracytoplasmic sperm injection.

3.2. *Transmission Electron Microscopy (TEM)*. Similarly, as by MSOME, transmission electron microscopy (TEM) revealed the sperm head vacuoles in normal and abnormal sperm samples. The vacuoles in sperm heads were seen as lighter areas inside the nucleus and were present in almost all spermatozoa in both types of sperm samples (Figures 3(a) and 3(b)). Electron micrographs of sperm head vacuoles are rarely presented in the literature and the interpretation of their ultrastructure is scarce [12, 25]. The description of fine structure of human spermatozoa usually presents large lucent areas in sperm heads as vacuoles and smaller clear areas as nucleoplasm or clear spots [26].

Contrary to light micrographs where vacuoles are seen as surface concavities, in electron micrographs, vacuoles

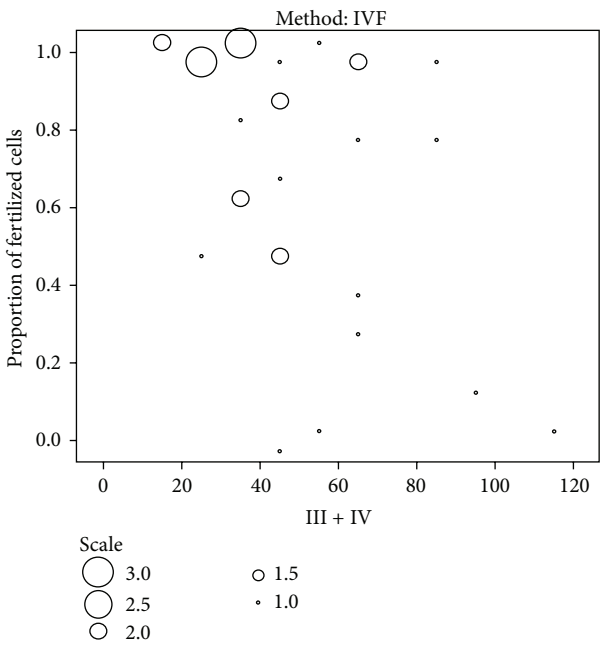


FIGURE 2: Relation between sperm head vacuoles (proportion of bad sperm: Class II plus Class III) in semen samples and oocyte fertilization rate after classical in vitro fertilization (IVF).

were occasionally observed as bulges at the cell surface (Figure 3(c)). Due to different optical density of vacuoles and condensed chromatin, nuclear indentations packed with membranes appear as surface concavities under light microscopy.

3.2.1. *Ultrastructural Features of Sperm Head Vacuoles*. Electron micrographs of sperm head vacuoles showed nuclear indentations containing stacks of membranes (Figures 4(c), 4(d), and 4(f)). These membranes were organized as concentrically arranged lamellae and are described as membrane whorls (MW), which occasionally bulge from the cell surface. Membrane whorls of various complexities were present in different areas of sperm nucleus and can occupy a large area of nucleus in both morphologically normal and abnormal spermatozoa. In almost all cases, membrane whorls were covered by intact acrosome and plasma membrane (Figures 4(c) and 4(d)). They were mostly composed of tightly packed double

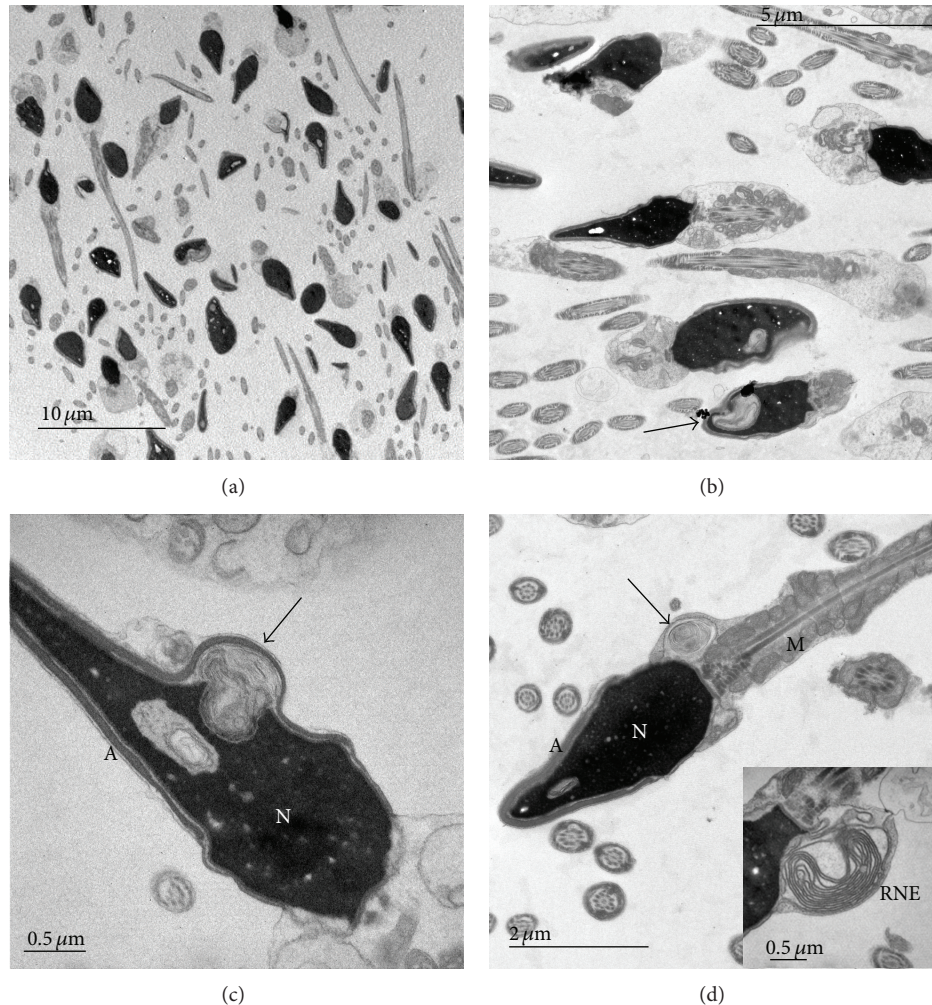


FIGURE 3: Transmission electron micrographs (TEM) of normozoospermic samples with head vacuoles of various size and position. Arrow indicates vacuoles on (b), (c), and RNE in (d). A: acrosome, N: nucleus, M: mitochondria, and RNE: redundant nuclear envelope.

membranes with electron dense molecular septa and thinner membranes enclosing flocculent material (Figures 4(d) and 4(f)). Franklin and Fussell [27] observed membrane whorls in apical subacrosomal space of late hamster spermatids which were continuous with the nuclear envelope. According to the authors [27], the membrane whorls appeared larger and were present in greater numbers in the final or maturation phase of spermiogenesis.

Sperm maturation leads to nuclear reorganisation and reduction of its size. From the literature, it is known that nuclear maturation involves reduction of nuclear envelope, a dynamic double membrane that surrounds the nucleus, segregates chromosomes, and regulates nuclear transport through pores [28]. Many details of its transformation are still unclear. Our micrographs of membrane whorls showed continuity of membranes with the nuclear envelope (Figures 5(a) and 5(c)). Membrane structures with septate electron dense molecular complexes are probably produced during the reorganization of the nuclear envelope. These structures were described by several authors as redundant nuclear envelope

(RNE) [11, 28, 29]. The RNE is seen in our micrograph of normal spermatozoa (Figure 3(d)).

Different stages of chromatin condensation are seen in our micrographs in all samples. In Figure 5(d), we can see one spermatozoon with abnormal morphology in a process of chromatin condensation which is still surrounded by large amount of cytoplasm. In late spermatogenesis, sperm DNA becomes tightly packed because histones are replaced by protamines [11]. However, condensation defects frequently occur and result in irregularly shaped clear spaces of various sizes in the nucleus [26]. Chromatin condensation failure is known to be associated with the presence of small and large vacuoles [2, 8]. Our micrographs revealed smaller electron lucent areas of noncondensed chromatin and nuclear vacuoles which differ in size and always contain amorphous material or/and membrane whorls. They might be more expressed in spermatozoa of teratozoospermic samples and it may be related to abnormal sperm maturation process and sperm head vacuoles. However, more focused research is required.

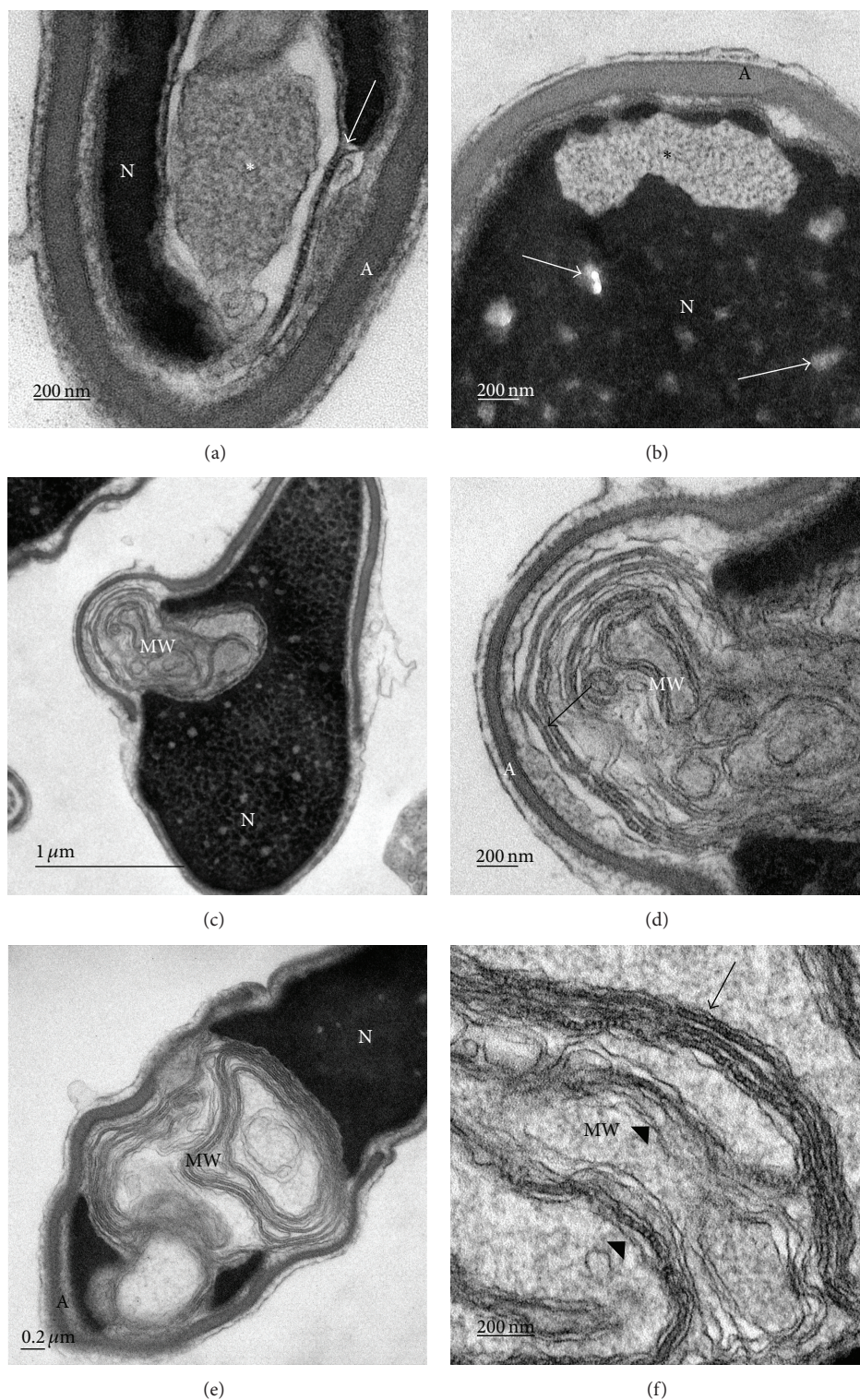


FIGURE 4: TEM micrographs of sperm head vacuoles of normozoospermia ((a), (c), (d), (e), and (f)) and teratozoospermia (b). (a) Nuclear indentations with membranes enclosing flocculent material (asterisk). Double membrane with molecular septa (arrow) emerging from nuclear envelope. (b) Nucleus with clear spots of noncondensed chromatin (arrow) and large area with flocculent material (asterisk). (c) Membrane whorls (MW) consisting of concentrically arranged membranes. (d) High magnification of membrane whorls of (c). (e) Membrane whorls (MW) occupying a large part of nucleus. (f) High magnification of double membranes with septal complexes (arrow) and thin membranes (arrow head). A: acrosome; N: nucleus.



FIGURE 5: Cross sections of spermatozoa in normozoospermic samples ((a), (b), and (c)) with large nuclear indentations with membrane whorls (MW). Acrosome membrane is connected with nuclear envelope (arrows). (c) Membrane whorls (MW) with additional membranes between nucleus and acrosome (arrowhead). (d) Large part of noncondensed chromatin with granular formation inside the nucleus (arrow) of spermatozoon with abnormal morphology. A: acrosome, N: nucleus, and C: cytoplasm.

Acrosome was fully developed in most spermatozoa with sperm head vacuoles. In some cells, interconnections of inner acrosomal membrane and nuclear envelope were observed (Figures 5(a) and 5(b)). Acrosome morphogenesis is a dynamic process that involves a close interaction between Golgi complex and nuclear envelope of early spermatid. It has been shown that perinuclear theca (PT) is a cytoskeletal structure that covers the sperm nucleus in mammals and has been shown to play important role in normal acrosome formation [30].

As reviewed by Setti et al. [31], some authors report that sperm vacuoles are of acrosomal origin [4] and reflect nonreacted acrosome [5]. A negative relation between the presence of vacuoles and the sperm capacity to undergo the acrosomal reaction is exposed. Our data show that the sperm head vacuoles are unique structures, usually covered by intact acrosome.

Abnormal spermatozoa were present in both types of sperm samples—normal and abnormal. Abnormalities

affected different components of the sperm cells, namely, chromatin, acrosome, neck region, mitochondria, and tail. We present several micrographs of abnormal spermatozoa from normozoospermic samples with less condensed chromatin, bended neck, atypical acrosome, and abnormal head shape (Figure 6).

3.2.2. Function of Sperm Head Vacuoles. At present, it is impossible to state what the real function of sperm head vacuoles is. Based on our micrographs of sperm ultrastructure, we suggest that sperm head vacuoles may remove some substance of unknown origin from chromatin. Because they indicate the potential relation to both the sperm maturation process in one side and abnormal chromatin condensation in the other, one may speculate that the sperm head vacuoles may be involved in the removal of histones when replaced by protamines. But it is too early for any conclusion and further research is needed.

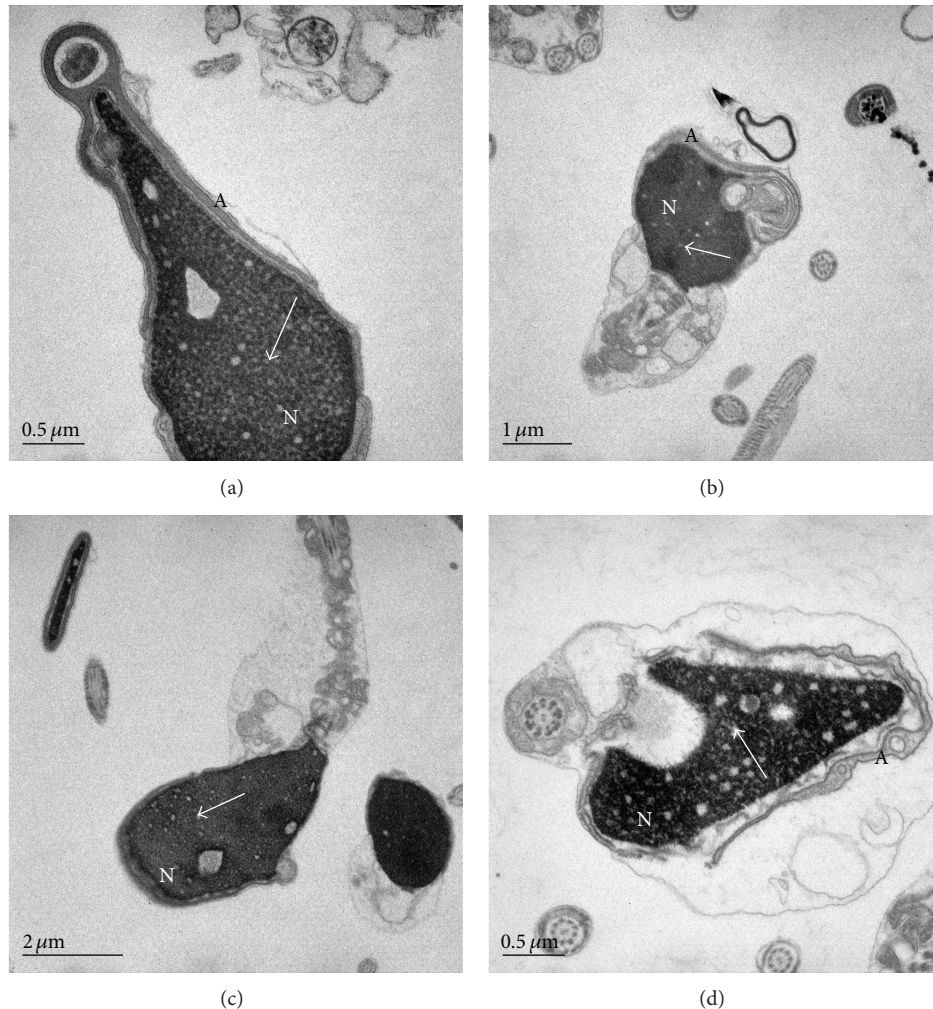


FIGURE 6: (a), (b), (c), and (d) Spermatozoa from normal sperm samples with abnormal morphology. Chromatin is granular and less condensed with numerous clear spots (white arrows). Acrosome is abnormally formed (a), partly formed (d), and sometimes connected to membrane whorls (b). (c) Bended neck region. A: acrosome; N: nucleus.

4. Conclusions

The results of this study indicate that sperm samples of men with teratozoospermia are characterized by increased ratio of sperm head vacuoles, although they are present also in sperm samples of normospermic men. This indicates that they may be related to pathological state. Our micrographs on sperm ultrastructure indicate that sperm head vacuoles are composed of membrane whorls. Variations in chromatin condensation were mostly observed in abnormal sperm samples and it is not excluded that they are related to the abnormal maturation and sperm head vacuoles. Further research combining different methods and transmission electron microscopy is needed to better elucidate this phenomenon.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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Research Article

High HPV Infection Prevalence in Men from Infertile Couples and Lack of Relationship between Seminal HPV Infection and Sperm Quality

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Human papillomaviruses (HPV) are the most frequently sexually transmitted viruses and etiological agents of several human cancers. Controversial results of the role of HPV in infertile population on sperm parameters have been published. The aim of this study was to estimate the type-specific prevalence of HPV DNA infection of the external genitalia and semen in 340 Slovenian men from infertile couples and to establish the relationship between seminal HPV DNA infection and abnormal sperm parameters. Self-taken swabs of the entire penile surface and semen samples were collected, and HPV detection and genotyping were performed. HPV DNA was detected in 37.12% of external genitalia and in 13.61% of semen samples with high HPV type concordance of both sampling sites. The most prevalent HPV types in the male external genitalia were HPV-CP6108 and HPV-84. The most prevalent HPV types in semen were HPV-53 and HPV-CP6108. The prevalence of HPV infection between normozoospermic men and men with abnormal sperm parameters did not differ significantly. Sperm quality did not differ significantly between men with seminal HPV infection and uninfected men. In conclusion, the men from infertile couples are equally susceptible to HPV infection regardless of their fertile potential; seminal HPV infection does not impair sperm quality.

1. Introduction

Human papillomaviruses (HPV) are the most common sexually transmitted viruses. More than three quarters of the sexually active human population will acquire an HPV infection during their lifetime, although most of the infections are self-limited [1]. Persistent HPV infection is a necessary cause of several human cancers, mainly in the anogenital area [2]. By February 2014, more than 179 HPV types have been officially recognized, and about 40 different HPV types from the clinically most important HPV genus (alpha) affect the anogenital region of both genders [3].

HPV can be detected in every part of the male reproductive tract [4–6]. Since the early reports of seminal HPV infection in the middle eighties, the role of HPV infection in infertility has been debated [7]. World Health Organization

(WHO) defines infertility as an inability of a sexually active, noncontraceptive couple to achieve spontaneous pregnancy in one year [8]. Approximately 15–20% of couples in reproductive period encounter infertility problems. Many factors affect the fertility potential and they are equally distributed between genders: in 1/3 of infertile couples, a male infertility associated factor is found, in 1/3 a female associated factor is found, and in 1/3 both female and male infertility associated factors are found present [9]. Maldescended testis, varicocele, and inflammation are among the most common diagnoses of male infertility and represent one of the three most frequent causes of male infertility [10]. In spite of highly improved diagnostic procedures, about 10% of couples remain diagnosed with idiopathic infertility that does not provide a cause for their defect [8]. A connection between asthenozoospermia and HPV DNA sperm infection has been

observed *in vivo* and *in vitro* [11–14]. Paradoxically, after unsuccessful sperm wash, when HPV DNA was still present in a sample, spermatozoa expressed higher motility [15]. However, the connection between a lower total sperm count and seminal HPV DNA infection has also been reported [16], whereas in another study the high-risk HPV (HR-HPV) sperm infection has been found to correlate to borderline lower pH of ejaculate [17].

HPV DNA possesses an ability of binding to the surface of viable spermatozoa [18–21]. Furthermore, viable spermatozoa have the potential of uptaking the exogenous HPV DNA fragments *in vitro* [22]. Lai et al. have shown the expression of oncogenes E6 and E7 of HPV-16 and HPV-18 separately in spermatozoa fraction and sperm plasma [23]. Foresta et al. have reported that transfected sperm is able to penetrate hamster oocytes [19]. *In vitro* studies have shown some detrimental effects of HPV DNA on sperm cells and early embryo development [24–26].

On the other hand, the absence of any significant correlation between seminal HPV DNA infection and abnormal sperm parameters has been reported [21, 27, 28].

The prevalence of HPV DNA seminal infection in patients attending fertility clinics ranges between 7.8% and 28.6% [21, 28]. There is a lack of published data on the prevalence of HPV infection in external genitalia in men from infertile couples.

In the Slovenian population, HPV has been studied for over 20 years [29], mostly in women. The overall prevalence of HR-HPV infection in women (mean age 36.6 years) in the recent national HPV Prevalence Study was 12.9%, whereas an 8.2% prevalence of HR-HPV types was assessed in 195 Slovene women undergoing the IVF program [30, 31]. However, there are no data on the prevalence of HPV infection in male sub/infertile population.

Recently, HPV has been proposed as an agent possibly impairing sperm quality and function. Due to inconsistency in reporting possible adverse effects of HPV infection on semen, we designed a study enrolling a representative sample population of male patients from the infertility clinic to screen their genital and seminal samples for HPV DNA. The aim of this study was to establish the type-specific prevalence of HPV DNA infection of external genitalia and semen with 37 different alpha HPV types in a group of men from Slovenian infertile couples. Additionally, relationships between the presence of HPV DNA in semen and abnormal sperm parameters were analysed.

2. Materials and Methods

2.1. Study Design and Population. This was a cross-sectional study conducted in an outpatient infertility clinic between October 2010 and October 2013. The study protocol had been approved by the National Medical Ethics Committee (Consent Number 53/09/10). Men were recruited prospectively at the same visit when they provided semen for sperm analysis. Men were eligible for participation if they (1) were male partners from couples attending the clinic for inability to conceive within at least one year of unprotected regular sexual intercourse, (2) underwent sperm parameters analysis, and

(3) provided semen by masturbation. Additionally, a clinical examination was done. Azoospermic men were excluded from the statistical analysis concerning the difference in sperm parameters between HPV infected and uninfected men.

2.2. Specimen Collection. After a written consent was obtained, the participants were carefully instructed to provide a semen sample and self-taken swab of the entire penile surface. They were provided with a sterile container for semen collection, a flocked nylon swab (MicroRheologics, Brescia, Italy) prewetted with saline for self-swabbing, and 1 mL of specimen transport medium (STM) (QIAgen, Gaitheburg, USA) for the swab storage. The participants were carefully instructed to wash their hands before masturbation and to swab the entire penile surface (avoiding possible sperm traces) immediately after masturbation. Self-swabbing using the prewetted swab took at least one minute to provide adequate number of exfoliated cells.

After the liquefaction of the collected semen samples, 100 μ L of semen samples and the entire STM with the inserted swab were frozen and stored at -20°C until PCR analyses and typing were undertaken. At the same time classical sperm parameters were analysed.

2.3. Sperm Analyses. Semen samples were provided by masturbation after 2–5 days of sexual abstinence. After an hour, semen was assessed according to the World Health Organization (WHO) 2010 guidelines by one of the two well-trained technicians [32]. Briefly, after initial macroscopic examination, a wet preparation was obtained for assessing microscopic appearance, sperm motility, and the dilution required for assessing sperm number. A standard volume of 10 μ L of semen was placed onto a clean glass slide and covered with a coverslip 24 mm \times 24 mm. When the sample was spread and the contents were no longer drifting, the progressive and nonprogressive motility was assessed with brightfield optics at $\times 400$ magnification. Many fields were viewed and the motility was displayed as an average proportion of instantly estimated sperm motility in those many fields. Afterwards, semen was diluted with fixative (1 : 5 or 1 : 20) for assessing sperm concentration. An improved Neubauer haemocytometer of 100- μ m-deep haemocytometer chambers was used. The method of feathering (the semen drop was spread along the back edge of the angled slide and pulled forwards over the slide to form the smear) was used for preparation of semen smears for assessing sperm morphology. The air-dried smears were fixed with ethanol and stained using the Papanicolaou procedure. The slides were examined with brightfield optics at $\times 1000$ magnification with oil immersion and 100 spermatozoa were assessed for the percentage of normal and abnormal forms. For sperm morphology evaluation, the strict criteria were used. The men were considered normozoospermic when sperm concentration was $\geq 15 \times 10^6$ spermatozoa/mL, progressive motility $\geq 32\%$, and the percentage of spermatozoa with normal morphology $\geq 4\%$ (representing the 5th centile, lower reference limits proposed by WHO 2010) [33]. The men

whose sperm concentration, motility, and the percentage of spermatozoa with normal morphology were below the lower reference limits were considered having abnormal sperm parameters.

2.4. Isolation of DNA. Total DNA was isolated from STM and semen samples using EZ1 Virus Mini Kit (QIAgen, Valencia, CA, USA) on BioRobot EZ1 workstation (QIAgen) according to the manufacturer's instructions.

DNA was eluted in the Elution buffer (QIAgen) and stored at 4°C.

2.5. PCR and HPV DNA Typing. HPV DNA amplification, detection, and typing were performed using the commercially available Linear Array HPV Genotyping Test (Roche Molecular Diagnostics, Pleasanton, CA) capable of detecting 37 different alpha-HPV types, following the manufacturer's instructions. Prior to PCR amplification using PCR GeneAmp 9700 (Life Technologies Corporation, Carlsbad, USA) concentrations of DNA in all isolates from semen samples were estimated by spectrophotometric analysis at 260 nm using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). 250 ng of DNA per final volume of 50 μ L PCR reaction was used. The isolates from swabbed samples were loaded in a maximum volume (25 μ L) per 50 μ L PCR reaction.

Hybridization and PCR detection were performed using automated processor ProfiBlot T48 (Tecan, Salzburg, AT). Types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 were considered as high-risk (HR) types [34]. Samples were considered valid if they were HPV-positive by genotyping or β -globin positive, regardless of HPV results. When HPV infection was subdivided on the basis of the risk of type of HPV, the men were assigned into a HR group, if they were infected with at least one HR type. Linear Array HPV Genotyping test uses multiple type probes (one cross-reactive oligonucleotide probe that hybridizes with HPV-33, HPV-35, HPV-52, and HPV-58) to detect DNA from HPV-52 infection; thus, we were unable to determine the HPV-52 status in the presence of HPV-33, HPV-35, or HPV-58 infection. In statistical analysis, such cases were considered as HPV-52 negative.

2.6. Statistical Analysis. Statistical analysis was performed using IBM SPSS Statistics 20 software (IBM Corporation, USA). General descriptive data of men with normozoospermia and abnormal sperm parameters and the values of sperm parameters were presented as arithmetic mean \pm standard deviation. Chi-Square statistic was used for the evaluation of differences in the HPV prevalence between normozoospermic men and men with abnormal sperm parameters. Mann-Whitney test was performed to compare sperm parameters between men with seminal HPV infection and uninfected men. Kruskal-Wallis test was performed to compare sperm parameters between HPV uninfected men, men with LR-HPV type of seminal infection, and men whose sperm was infected with at least one of HR-HPV. When the difference was significant, linear regression was performed, and age, abstinence, number of leukocytes, orchiopexy, testicular

TABLE 1: Mean values of characteristics of men from infertile couples, $n = 340$.

	Normozoospermia, $n = 246$	Abnormal sperm parameters, $n = 94$
Age (years)	33.2 \pm 4.8	32.2 \pm 5.9
Duration of infertility (years)	1.9 \pm 1.0	1.9 \pm 1.5
Smoking (number of cigarettes per day)	4.6 \pm 6.7	4.6 \pm 8.4
Testicular volume-left (mL)	20.6 \pm 4.1	16.0 \pm 6.0
Testicular volume-right (mL)	21.2 \pm 4.2	16.1 \pm 6.5
Abstinence (days)	4.4 \pm 3.8	3.7 \pm 1.5
Sperm volume (mL)	3.3 \pm 1.3	3.6 \pm 1.7
pH	7.7 \pm 0.2	7.8 \pm 0.2
Motility a + b (%)	53.0 \pm 7.0	26.6 \pm 18.4
Concentration (spermatozoa/mL)	74.4 \pm 40.2	13.3 \pm 17.7
Total sperm count	241.4 \pm 161.5	42.9 \pm 64.7
Spermatozoa with normal morphology (%)	24.6 \pm 12.8	7.8 \pm 11.8
Leukocytes (per mL)	0.68 \pm 1.2	0.75 \pm 1.0

trauma, varicocele, and testicular volume were used as covariables.

3. Results

3.1. Population and Samples Adequacy. Overall, 340 men were enrolled, their mean age being 32.91 \pm 5.15 years. Mean values of age, duration of infertility, number of smoked cigarettes per day, testicular volume, and sperm parameters for a group of men with normozoospermia and a group of men with abnormal sperm parameters are summarized in Table 1. Forty-one samples of swabbed penile surface and 24 semen samples were not provided properly or the amplification of internal control (β -globin) failed.

3.2. Prevalence of HPV DNA Infection of Penile Surface. The prevalence of HPV DNA infection of the penile surface was 37.12% (111/299). The most prevalent HPV types on the penile surface were HPV-CP6108 and HPV-84 with type-specific prevalence 5.35% each, followed by HPV-53, HPV-16, HPV-62, and HPV-54, each detected in 4.01%–4.68% of samples (Figure 1, Table 3). Prophylactic vaccine types HPV-16, HPV-18, HPV-6, and HPV-11 were detected in 4.35%, 2.01%, 3.34%, and 0% of the penile surface samples, respectively. 45.45% of all HPV infected men presented HR-HPV in samples of the swabbed penile surface; 45.95% of all detected HPV DNA infections were multitype infections.

3.3. Prevalence of HPV DNA Infection in Semen. The prevalence of HPV DNA infection in semen was 13.61% (43/316). The most prevalent types in semen were HPV-53 and HPV-CP6108 with the type-specific prevalence of 2.85% and 2.53%,

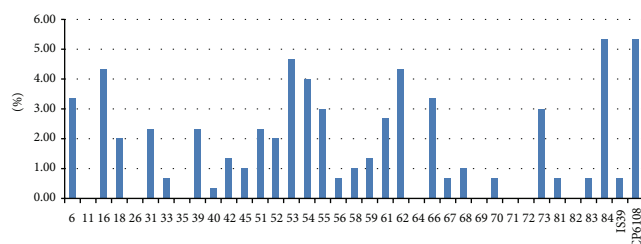


FIGURE 1: Type-specific prevalence of HPV infection of penile surface in men from infertile couples ($n = 299$).

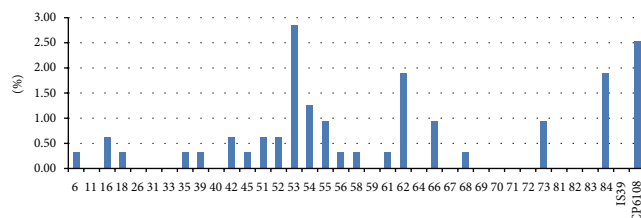


FIGURE 2: Type-specific prevalence of HPV infection of semen in men from infertile couples ($n = 316$).

respectively, followed by HPV-84, HPV-54, and HPV-62, each detected in 1.27%–1.90% (Figure 2, Table 4). Prophylactic HPV-16, HPV-18, HPV-6, and HPV-11 vaccine types were detected in 0.63%, 0.32%, 0.32%, and 0% of the semen samples, respectively. In three of the four seminal infections, low-risk HPV (LR-HPV) types were present. Multitype infection was observed in 27.91% of all infections.

3.4. HPV DNA Infection in Normozoospermic Men versus Men with Abnormal Sperm Parameters. Men from the total cohort were subdivided according to sperm parameters in a normozoospermic group and a group of men with abnormal sperm parameters; 246 (72.4%) men were considered normozoospermic, and 94 (27.6%) men had abnormal sperm parameters. Semen analysis of 4.1% (14/340) of men showed azoospermia, and 23.5% (80/340) of men had combined oligoasthenoteratozoospermia (OAT). Oligozoospermia and/or asthenozoospermia and/or teratozoospermia, asthenozoospermia and/or oligozoospermia and/or teratozoospermia, and teratozoospermia and/or oligozoospermia and/or asthenozoospermia were observed in 71, 52, and 42 men, respectively. Isolated oligozoospermia, asthenozoospermia, and teratozoospermia were observed in 30 (8.8%), 13 (3.8%), and 6 (1.8%) men, respectively. No significant difference in seminal HPV DNA infections was observed between the normozoospermic group and the group of men with abnormal sperm parameters (Table 2).

3.5. Concordance of HPV Type Infection among Genital and Seminal (Paired) Samples of the Same Men. Concordance of at least one of the detected HPV types was found in 30.51% (36/118) of paired samples. In 58.47% (69/118) of paired samples, only one of the two checked samples contained HPV DNA. We were unable to determine the concordance

in 11.02% (13/118) of paired samples, since one of the paired samples was HPV-positive and the other was inhibited. The negative concordance for all types was 50.59%. The total HPV type-specific concordance was 60.59%.

3.6. The Effect of Seminal HPV DNA Infection on Sperm Parameters. No statistically significant difference was found in sperm quality between men with seminal HPV infection and uninfected men. The difference in sperm quality between HPV uninfected men and men with HR- or LR-HPV seminal infection was not statistically significant.

4. Discussion

The relationship between HPV infection in men and abnormal sperm quality is controversial. As long as routine sperm washing fails to eliminate HPV DNA from the ejaculate, it is of crucial importance to determine whether HPV DNA sperm infection affects the quality of sperm parameters or not [16, 35]. The aim of this study was to establish the type-specific prevalence of HPV DNA infection of external genitalia and semen with at least one of the 37 most important alpha HPV types in a group of men from Slovenian infertile couples. Additionally, relationships between the presence of HPV DNA in semen and abnormal sperm parameters were studied.

4.1. Genital Surface HPV Prevalence. The sample size of the study population was calculated prior to recruitment, the men were recruited systematically, and the selection bias was minimal; therefore we may consider our study sample representative. We found the overall prevalence of HPV infection in men from infertile couples to be 40.28%, regardless of the sampling site and sperm quality. Since the penile surface (shaft, prepuce, frenulum, coronal sulcus, and glans) is frequently infected with HPV, our first aim was to determine the genital HPV prevalence in men from infertile couples [36]. The obtained prevalence of HPV infection of the penile surface was 37.03%. Since men underwent semen analysis after two to five days of sexual abstinence, we assume that the detected infection was not a contamination from their female partners. Self-swabbing for a minute and immediately after masturbation is supposed to increase the prevalence. Self-swabbing has been reported to be as efficient as sampling by a physician [37]. The detection of an active infection, without a possibility of acquiring new or reactivated infection at a delayed visit, was assured by collecting samples from the penile surface and semen at the same visit [38]. It is difficult to make direct comparisons of the genital HPV prevalence between the published studies. To the best of our knowledge, this is the first study reporting the genital HPV prevalence in men from infertile couples. HPV prevalence among men has been reported to range from 1.3% to 72.9% [1]. In a recent study from Florida dealing with heterosexual couples aged 18–70 years, the genital HPV prevalence in males for any type was 55.7% [39]. In the HPV in Men Study that enrolled men having sex with men, men having sex with women and men, and men having sex with women (MSW), the genital HPV prevalence among MSW was 42% with the peak in the group

TABLE 2: Prevalence of seminal HPV DNA infection in men from infertile couples according to their sperm parameters.

	Normozoospermia	Abnormal sperm parameters				<i>p</i> value
		O	A	T	OAT	
HPV DNA infection of semen	24/160 (15.0%)	2/24 (8.3%)	1/11 (9.1%)	1/6 (16.7%)	3/30 (10.0%)	NS

O: oligozoospermia; A: asthenozoospermia; T: teratozoospermia; OAT: oligoasthenoteratozoospermia; NS: not significant ($p > 0.05$).

TABLE 3: Type-specific prevalence of HPV in samples from penile surface ($n = 297$).

HPV type	Type-specific prevalence	Number
CP6108	5.35%	16
84	5.35%	16
53	4.68%	14
62	4.35%	13
16	4.35%	13
54	4.01%	12
66	3.34%	10
6	3.34%	10
73	3.01%	9
55	3.01%	9
61	2.68%	8
51	2.34%	7
39	2.34%	7
31	2.34%	7
52	2.01%	6
18	2.01%	6
59	1.34%	4
42	1.34%	4
68	1.00%	3
58	1.00%	3
45	1.00%	3
IS39	0.67%	2
83	0.67%	2
81	0.67%	2
67	0.67%	2
56	0.67%	2
33	0.67%	2
70	0.67%	2
40	0.33%	1
72	0.00%	0
71	0.00%	0
69	0.00%	0
64	0.00%	0
82	0.00%	0
35	0.00%	0
26	0.00%	0
11	0.00%	0

TABLE 4: Type-specific prevalence of HPV in semen samples ($n = 316$).

HPV type	Type-specific prevalence	Number
53	2.85%	9
CP6108	2.53%	8
62	1.90%	6
84	1.27%	6
54	1.27%	4
55	0.96%	3
66	0.96%	3
73	0.96%	3
16	0.63%	2
42	0.63%	2
51	0.63%	2
52	0.63%	2
6	0.32%	1
18	0.32%	1
35	0.32%	1
39	0.32%	1
45	0.32%	1
56	0.32%	1
58	0.32%	1
61	0.32%	1
68	0.32%	1
11	0.00%	0
26	0.00%	0
31	0.00%	0
33	0.00%	0
40	0.00%	0
59	0.00%	0
64	0.00%	0
67	0.00%	0
69	0.00%	0
70	0.00%	0
71	0.00%	0
72	0.00%	0
81	0.00%	0
82	0.00%	0
83	0.00%	0
IS39	0.00%	0

aged 25–34 years [40]. Another recent study reported a 41.7% HPV DNA prevalence in a cohort of 1033 males (median age 34 years) who were screened for sexually transmitted diseases, investigation of suspected HPV-related lesions, or because of HPV-positive partners [41]. With respect to the

latter, genital HPV prevalence in men from infertile couples seems to be particularly similar to the genital HPV prevalence among the general age-related population of MSW, even if the population of infertile couples could be speculated to differ in sexual behaviour and promiscuity from general heterosexual

couples. The mean duration of infertility in couples of participants in our study was almost two years, supposing that the duration of their stable sexual relationships was even longer. Taking into account the clearance time of HPV infection, which is generally shorter than two years in men, we expected much lower HPV prevalence [42].

The most prevalent HPV types in the penile surface samples of men from infertile couples were HPV-84 and HPV-CP6108. The latter is a LR-HPV type and one of the most frequently detected HPV types in men [36], but rarely present in women [41]. HPV-84 is a HPV type without clearly established clinical significance that has a relatively high prevalence [6, 36, 41]. Half of the HPV infections of the penile surface were caused by LR-HPV types. Interestingly, in contrast to HPV-6, HPV-11 was not detected in any sample tested.

4.2. Seminal HPV Prevalence. The prevalence of seminal HPV infection differs greatly between different groups of men, from 10.2% in infertile men to 53.8% in men with genital warts [14]. In our study, seminal HPV infection was detected in 13.38% of men from infertile couples. The prevalence is in concordance with that established in similar studies in which men from infertile couples were enrolled [14, 21]. The most frequent types detected were HPV-53 and HPV-CP6108, followed by HPV-84, HPV-54, and HPV-62, respectively. Comparative evaluation of the type-specific HPV DNA prevalence of the penile surface and concordant semen samples showed that the most frequent HPV types on the penile surface were also among the most frequent types and, moreover, in almost the same order as in semen samples. High concordance of at least one of the detected HPV types in both samples might be explained by the observation provided by a study performed in France in 2002, where patients with a positive HPV semen sample and penile or urethral lesions had the same HPV type detected in the two specimens [43]. In our study, almost one-third of men had the same HPV type on the penile surface and in the semen. It is widely known that the preferred target of HPV is the squamous epithelium [44]. Since squamous epithelium is present in the male reproductive tract only in the distal 2 cm of the urethra, this could be a possible source of seminal infection/contamination with the same type as detected in a penile surface sample [45]. If HPV might affect the sperm quality, the virus should not only contaminate but also cohabit with spermatozoa under certain conditions [18]. Multitype infections were less likely to be detected in seminal samples than in penile surface samples. Three of the four HPV types detected in semen were LR-HPV types. The latter might suggest a selective mechanism of the seminal HPV infection.

Slovenia has integrated the HPV vaccination into its national immunization program in 2009 and currently provides routine vaccination free of charge only to the primary target population (11- to 12-year-old girls) [46]. The prevalence of the HPV types covered by quadrivalent vaccine is expected to decline among primary target population in the next decades; however, current HPV national vaccination program does not affect infertile couples. Almost half of the HPV-infected men in our study had at least one of the HR-HPVs on the swabbed penile surface. Accordingly, the great majority of female partners of (at least) the HPV-infected

men are supposed to be nonnaïve for specific HPV types, if not also currently infected [39]. Although not covered by government, it will be worthy to offer HPV vaccination to all females from infertile couples, at least to partners of HPV-negative males.

4.3. HPV Infection in Men with Different Sperm Quality. Male reproductive capacity was found to be deficient in not less than 50% of infertile couples evaluated [8, 9]. By the new "WHO laboratory manual for the examination and processing of human semen," normozoospermia is defined as sperm concentration, motility, and morphologically normal spermatozoa that are equal to or above the lower reference limits. When the 1999 WHO reference values were used, approximately 40% of men from infertile couples were reported to have an abnormal sperm parameter [33]. The introduction of the new WHO reference values in 2010 resulted in 15% of infertile men being reclassified as fertile, if the status is based on semen analysis alone [47]. In a recent study, the proportion of sperm abnormalities (pH, sperm volume, concentration, total sperm count, and motility) was compared between WHO 1999 and WHO 2010 classification: abnormal sperm parameters according to WHO 2010 reference values were found only in 25.4% of 571 men from couples undergoing fertility investigations [48]. However, in our study, 27.6% of men from infertile couples could have been considered infertile according to the WHO 2010 reference values of sperm analysis. Interestingly, converting the WHO 2010 to WHO 1999 reference values would bring 168 (49.4%) men from our study having abnormal sperm parameters. For men from infertile couples, it seems important to know whether the seminal HPV infection affects their sperm potential or not, regardless of the more or less arbitrary reference values. The number of men with isolated sperm defects was too low to allow for a credible statistical analysis, but the prevalence of HPV infection of the penile surface and semen in the total group of men that had abnormal sperm parameters was not significantly different from that in normozoospermic men (not even, if WHO 1999 reference values would have been used).

In statistical analysis, when HPV infection prevalence was compared between normozoospermic men and the total group of men with abnormal sperm parameters, men with azoospermia were excluded. However, it is interesting to note that four azoospermic men were diagnosed with the HPV DNA infection of the penile surface, one of them having additional HPV infection in the ejaculate. All of them had histologically confirmed nonobstructive azoospermia (Sertoli cell-only syndrome with early maturation arrest). The presence of HPV DNA in testicular biopsies from nonobstructive azoospermic men had previously been reported, but there is lack of data on HPV DNA presence in ejaculated sperm [5]. In the sample of ejaculate of only azoospermic men with HPV infection of the penile surface and semen, HPV-CP6108 was detected. It is possible that the source of the detected seminal type was contamination from the genital surface, where multitype infection with HPV-6, HPV-39, HPV-51, and HPV-CP6108 was detected. However, the presence of infected nonsperm cells in the ejaculate could not be ruled

out. Therefore, it seems that all men are equally susceptible to HPV infection, regardless of their fertile potential and that seminal HPV infection does not impair sperm quality.

4.4. The Difference in Sperm Parameters between HPV Infected and Uninfected Men. In the present study, we were unable to detect any significant difference in a variety of sperm parameters previously reported as impairing the sperm quality, between men having seminal HPV infection and uninfected men. The results of our study imply that HPV infection does not seem to affect sperm quality in terms of causing clinically significant alterations of sperm parameters. Similar results have been demonstrated in a study involving couples undergoing IVF treatment and were screened for the HPV-16 DNA infection [27]. Moreover, semen samples of men seeking fertility evaluation were screened for any type of HPV infection [28]. The prevalence of seminal infection in the latter study was higher than in the present study; however, no significant difference in sperm quality was found between infected and uninfected men. In a recent study, 308 semen samples of male partners of couples undergoing IVF treatment were screened for the HR-HPV DNA infection [21]. The HPV DNA infection did not significantly differ between infected and uninfected men. In our study, we have not observed any clinically significant alteration of sperm parameters, which is contrary to some authors who have reported an association between seminal HPV infection and decreased sperm motility or reduced pH of seminal plasma [11, 13, 16, 17]. The studies reporting on the differences in sperm quality between infected and uninfected men are inconsistent, not providing useful information on which sperm parameter would be most indicative of HPV infection. Furthermore, there have been some opposite effects of seminal HPV infection on sperm parameters reported; some groups have demonstrated decreased sperm motility in HPV infected men, whereas the *in vitro* studies have found that normal spermatozoa have higher motility after incubation with specific HR-HPV DNA [15, 49]. A possible explanation for these discrepancies may be a small and selected study size, narrow range of tested HPV types, or coinfection with other urogenital infections.

5. Conclusion

To the best of our knowledge this is the first study where the genotype-specific prevalence of HPV DNA infection of external genitalia of men from infertile couples has been estimated. HPV DNA has been detected in more than one-third of external genitalia samples and one-eighth of semen samples in men from infertile couples with a mean duration of infertility of almost two years. The HPV type-specific concordance of genital and seminal infection exceeds 60%. No significant differences in the prevalence of HPV DNA infection between groups of normozoospermic men and men with abnormal sperm parameters have been found. Similarly, we have not found any significant difference in sperm quality between the men with seminal HPV infection and uninfected men. The results of our study suggest that the presence of HPV does not impair sperm quality.

Conflict of Interests

All authors declare that there is no conflict of interests regarding the research, authorship, and/or publication of this paper. The authors inform that all materials and trademarks mentioned were used just as part of experimental protocol and there are no financial gains or favouritism of business.

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Review Article

Proteomes of Animal Oocytes: What Can We Learn for Human Oocytes in the *In Vitro* Fertilization Programme?

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Oocytes are crucial cells for mammalian reproduction, yet the molecular principles underlying oocyte development are only partially understood. Therefore, contemporary proteomic approaches have been used increasingly to provide new insights into oocyte quality and maturation in various species such as mouse, pig, and cow. Especially, animal studies have helped in elucidating the molecular status of oocytes during *in vitro* maturation and other procedures of assisted reproduction. The aim of this review is to summarize the literature on mammalian oocyte proteome and secretome research in the light of natural and assisted reproduction and on lessons to be learned for human oocytes, which have so far remained inaccessible for proteome analysis.

1. Introduction

The oocyte is one of the crucial cells in the live organisms, which enables the reproduction and continuation of the species. Because the main role of an oocyte is to be fertilized and to grow further into a fully functional organism, it needs to regulate many different cellular and developmental processes, such as cellular metabolism, cell cycle progression, fertilization, activation of zygotic transcription, embryo development, activation of the embryonic genome, and formation of embryonic axes [1–6]. During oocyte growth and maturation a variety of maternally transcribed mRNAs accumulate, representing the maternal contribution to the oocyte and, consequently, the newly fertilized oocyte, zygote, and early embryo [7–9]. The majority of these mRNAs are stored in message ribonucleoprotein (mRNP) complexes and are only translated when needed at specific stages of maturation [10]. In addition, they can be localized within a specific region of the cytoplasm or as being dispersed within the cytoplasm of the entire oocyte. Early embryonic development before the activation of embryonal genome is directed by maternal mRNAs expressed in oocytes and stored in mRNPs and occurs in the mid two-cell stage in the mouse,

the four-cell stage in the pig, the eight-cell stage in the sheep, and between the four- and eight-cell stages in humans [11]. The real proportion of genes expressed in oocytes is still poorly understood but it is clear that translational activity and its regulation are crucial for oocyte development and maturation [12].

Indeed, proteomic approaches are potentially very powerful to elucidate critical aspects of oocyte development or quality (Figure 1), but this is a largely unexplored territory. Studies over the last ten years have studied oocyte proteomes of various mammalian species, however largely excluding human oocytes due to technical issues (e.g., collection of sufficient number of oocytes), lack of access to cells, or to ethical concerns. The aim of this review is to summarize the literature on animal oocyte proteome and secretome studies to elucidate what can be learned for human oocytes in the *in vitro* fertilization programme.

2. Proteomics of Animal Oocytes

Most of the oocyte proteomic studies were performed in the mammalian species, especially in mouse, bovine, and

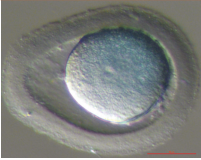





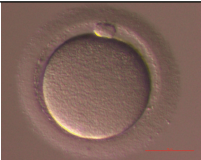

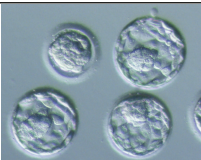



Potential focuses of oocyte proteomics	
	 <ul style="list-style-type: none"> Oocyte quality Oocyte morphology Female infertility Ovarian infertility
	 <ul style="list-style-type: none"> Oocyte-cumulus cells communication Secretion
	 <ul style="list-style-type: none"> Oocyte immaturity
	 <ul style="list-style-type: none"> Oocyte maturation Oocyte <i>in vitro</i> maturation
	 <ul style="list-style-type: none"> <i>In vitro</i> fertilization (IVF, ICSI) Ovarian hormonal stimulation and oocyte status Oocyte slow-freezing Oocyte vitrification Oocyte thawing
	 <ul style="list-style-type: none"> Oocyte DNA methylation Epigenetics Reprogramming Embryogenesis Embryonal development

FIGURE 1: Some potential focuses of proteomics to study human oocytes.

porcine models. In all these studies oocytes were aspirated from ovarian follicles by mostly 18-gauge needle attached to a sterile syringe or vacuum system from the animals with or without pretreatment with gonadotropins. In some studies oocytes were aspirated from isolated ovaries to retrieve sufficient numbers of oocytes to be analyzed. Typically samples consisted of several hundreds to several thousands of oocytes. In some studies the zona pellucida was removed from oocytes by enzyme or acid solution to possibly increase the number of detected proteins in the cytoplasm, nucleus and oolemma but in most studies intact oocytes were analyzed. Despite continued progress in proteomic technologies, in particular mass spectrometry, maximizing the number of available cells is of critical importance to reach sufficient proteome depth. In the study of Wang et al. [13] an impressive number of 7,000 mouse oocytes at different developmental stages were analyzed using semiquantitative mass spectrometry, identifying 2,781 proteins in immature oocytes, 2,973 proteins in mature oocytes, and 2,082 proteins in fertilized oocytes (zygotes).

Because of the large number of analyzed oocytes, this study provided a deep insight into the protein expression profile of mouse oocytes. This has demonstrated that oocytes are quite “active” cells expressing proteins related to a range of biological functions such as protein metabolism, transport, cell cycle and proliferation, stress response, developmental processes, RNA and DNA metabolism, cell organization and biogenesis, cell-cell signaling, signal transduction, and cell adhesion [13]. Yet, the number of detected proteins was significantly lower than the number of transcripts (approximately 16,457 genes) in mature mouse oocytes analyzed by SOLiD whole transcriptome analysis [14]. Comparison of protein expression profiles of mouse oocytes to mouse embryonic stem cells (mESCs) showed high similarity between immature and mature oocytes on one hand, and between fertilized oocytes (zygotes) and mESCs on the other hand [13] which may be explained by the activation of the molecular programme in oocytes transiting into the embryo.

In other proteomic studies fewer oocytes were analyzed resulting in lower numbers of detected proteins. Yet, as further discussed below, they have elucidated important relations between oocyte proteome composition and oocyte quality, maturity, *in vitro* maturation, and other *in vitro* fertilization procedures.

3. Proteomics and Oocyte Quality

In the *in vitro* fertilization programme, ovaries of women are hormonally stimulated to retrieve more oocytes for fertilization and for treatment of severe infertility. Little is known about oocyte quality admitted in the *in vitro* fertilization programme, where oocyte morphology is almost the only criterion used in daily medical practice. Therefore, in the absence of objective (molecular) criteria for oocyte quality, it is almost impossible to predict the outcome of *in vitro* fertilization treatment. Indeed, much more has been learnt from protein expression profiling either directly from human oocytes or by inferring such insight from animal oocytes.

Among very few studies on this topic, Powell et al. [16] aimed to discover putative quality markers of pig oocytes by comparative proteomics using differential labeling of proteins by ExacTags, followed by quantitative tandem mass spectrometry. Pig oocytes can be obtained in much larger quantities than rodent or nonhuman primate oocytes. In addition, they provide an excellent biomedical model as they mimic some of the events of oocyte maturation and early development in humans. Pig oocyte extracts (oocyte proteome) from groups of 100 oocytes and pig oocyte-conditioned *in vitro* maturation media (oocyte secretome) were obtained from high- and low-quality pig oocytes in terms of their developmental potential in TCM-199 medium with or without added gonadotropins FSH and LH. Sixteen abundant proteins were identified in the oocyte proteome that were differentially expressed in high- and low-quality oocytes (see Table 1). Proteins that were more abundant in the proteome of high-quality oocytes included kelch-like ECH-associated protein 1 (an adaptor for ubiquitin-ligase CUL3), nuclear export factor CRM1 (controlling the movement of

TABLE 1: Proteins that are differently expressed in the proteomes of high- and low-quality pig oocytes [16].

Differently expressed proteins in low- and high-quality pig oocytes
Upregulated in high-quality oocytes
Glutaminase (GLS)
Hypothetical protein Gi/24528344
Nuclear export factor CRM1 (CRM1)
Kelch-like ECH-associated protein 1 (KEAP1)
IS10-right transposase
Albumin
Ryanodine receptor (RyR)
Cortactin-binding protein 2 (CTTNBP2)
Fatty acid synthase (FASN)
Epidermal growth factor (EGF)-receptor (EGFR)
Ataxia-telangiectasia mutated protein (ATM)
Upregulated in low-quality oocytes
β -1 adrenergic receptor (ADRB1)
25-Hydroxyvitamin D3 1 α -hydroxylase
Connective tissue growth factor (CTGF)
Dystrophin (DMD)
Mannose-6-phosphatase/IGF II receptor

DNA methyltransferase 1 (DNMT1) and maintenance of DNA methylation patterns in the early embryo), and ataxia-telangiectasia mutated protein kinase (may be involved in DNA repair). Interestingly, low-quality oocytes secreted monoubiquitin as well as several proteins implicated in human disease, such as dystrophin (DMD) and cystic fibrosis transmembrane conductance regulator (CFTR), two proteins implicated in muscular dystrophy, and cystic fibrosis, respectively. It was concluded that quantitative proteomic analysis of limited samples sizes can suffice to identify potential markers reflecting oocyte quality, which might also be applied for the detection of biomarkers of human oocytes noninvasively.

4. Proteomics and Oocyte Maturity

Oocyte maturation is a complex process consisting of a cascade of molecular events leading to the marking of the following generation, and proper progression through these stages is critical to the success of assisted reproduction techniques (ART) in humans and other mammals [17–21]. In the *in vitro* fertilization programme the majority of oocytes retrieved from ovaries by ultrasound-guided aspiration of follicles is mature, that is, at the metaphase II (MII) stage, and thus can be fertilized. But there is also a proportion of immature oocytes at the prophase I/germinal vesicle (GV) or metaphase I (MI) stage that is refractory to fertilization. Since immature oocytes decrease the female fertility and impair the *in vitro* fertilization outcome, it is important to have insight into oocyte maturity. Proteomic approaches may help to identify proteins that reflect oocyte maturation state with

important implications for female meiotic maturation and further embryonic development.

4.1. Proteomes of Immature and Mature Oocytes. Proteomic studies of oocytes at different stages of maturation using tandem mass spectrometry revealed the expression of transforming acidic coiled coil containing protein (TACC3) in mouse immature GV-stage oocytes [22]. This protein plays a role in the microtubule-dependent coupling of the nucleus and the centrosome and is a motor spindle protein that may play a role in stabilization of the mitotic/meiotic spindle. It may be involved in the control of cell growth and differentiation and may contribute to cancer. In situ hybridization of mouse ovarian tissue sections displayed abundant expression of TACC3 specifically in the cytoplasm of growing oocytes but not in the primordial or atretic follicles. This pattern of expression indicated that in the mouse TACC3 is expressed in ovarian cells undergoing active growth and development.

On the other hand, proteomics revealed a group of highly abundant heat shock proteins and molecular chaperones in the mature MII-stage mouse oocytes and their localization on the plasma membrane, that is, the oolemma [23]. This suggests that heat shock proteins and molecular chaperones are not only involved in their “usual actions” such as oocyte response to environment by regulation of apoptosis (defense mechanisms) and protein folding but may also play a role in the process of oocyte maturation.

In a comparison of mouse GV and MII-stage oocytes, Vitale et al. [24] used two-dimensional (2D) electrophoresis and mass spectrometry identifying 500 proteins, 12 of which were differentially expressed between these stages (Table 2). Similar to the studies mentioned above these proteins also include TACC3 protein and heat shock proteins (HSP105, stress-inducible phosphoprotein STIP1 that acts as an adaptor protein coordinating the functions of HSP70 and HSP90). Among these proteins were also two epigenetics-related proteins: nucleoplasmin 2 (NPM2; essential for nuclear and nucleolar organization and early embryonic development) and spindlin (SPIN1; a major maternal protein expressed in the mouse during the transition from oocyte to embryo). In addition, it was found that NPM2, SPIN1, programmed cell death six-interacting protein (PDCD6IP), and importin alpha2 may be posttranslationally modified by phosphorylation during oocyte maturation [24]. Prompted by the fact that NPM2 is an oocyte-restricted protein related to epigenetic regulation, further investigations into its properties during oocyte maturation and preimplantation development revealed that NPM2 mRNA levels rapidly decline at fertilization. Indirect immunofluorescence analysis showed that, with the exception of cortical localization in mature MII-stage oocytes, NPM2 is localized in the nucleus of GV-stage oocytes and at all stages of preimplantation embryos.

Cao et al. [25] used a similar approach using 2-DE methodology identifying 63 proteins differentially expressed between mouse GV and MII-stage oocytes after removal of the zona pellucida (see Table 2). Six of these proteins overlapped with those in above mentioned study of Vitale et al. [24] (e.g., NPM2, SPIN1, adenylosuccinate synthetase

TABLE 2: Selected proteins that are differently expressed in immature and mature mouse oocytes according to [24, 25].

Some of proteins differently expressed in immature and mature mouse oocytes
Vitale et al. [24]:
Transforming acidic coiled coil containing protein (TACC3)
Heat shock protein 105 (HSP105)
Programmed cell death six-interacting protein (PDCD6IP)
Stress-inducible phosphoprotein (STIP1)
Importin alpha-2 (KPNA2)
Adenylosuccinate synthase (ADDS)
Nudix
Spindlin (SPIN)*
Lipocalin (LCN)
Lysozyme
Translationally controlled tumor protein (TCTP)
Nucleoplasmin 2 (NPM2)*
Cao et al. [25]:
Methionine adenosyltransferase II beta (MAT2B)
Proteasome (prosome, macropain) 26S subunit ATPase 6 (PSMC6)
Inositol-3-phosphate synthase 1 (ISYNA1)
Eukaryotic translation initiation factor 5A (EIF5A)
Protein-arginine deiminase type-6 (PADI6)*
CTP synthase (CTPS)
Adenylosuccinate synthase (ADDS)
Nucleoplasmin 2 (NPM2)*
Zinc finger, BED-type containing 3 (ZBED3)
Lambda-crystallin homolog (CRYL1)
Guanine deaminase (GDA)
Insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP2)
Nudix (nucleoside diphosphate linked moiety X)-type motif 5 (NUDT5)
Spindlin 1 (SPIN1)*

Legend: * highly important (maternal effect proteins related to epigenetics).

related to purine biosynthesis, and Nudix). Among differently expressed proteins were 21 proteins which were decreased or even absent in MII-stage oocytes, while 33 proteins were more abundant in mature MII-stage oocytes. Interestingly, three of these proteins were maternal effect proteins related to epigenetics: NPM2, SPIN1, and protein-arginine deiminase type-6 (PADI6). The differently expressed proteins showed significant enrichment for four pathways: purine metabolism, proteasome, glycolysis and gluconeogenesis, and pyruvate metabolism and were related to meiosis, fertilization, and regulation of early embryo development.

Some other studies showed that also protein phosphorylation changed during oocyte maturation. In the study of Ma et al. [26], two-dimensional electrophoresis (2-DE) of mouse MII-stage oocyte proteins and staining with silver staining or Pro-Q Diamond dye was performed to describe the proteome

and phosphoproteome of mouse oocytes. A total of 380 unique proteins were identified, in which 90 protein spots representing 53 (14%) unique proteins were stained with Pro-Q Diamond thus suggesting that they were phosphorylated. Moreover, Pelech et al. [27] investigated the regulation of protein kinases, phosphatases, and other regulatory proteins during meiotic maturation of pig immature GV-stage oocytes to maturing MI-stage oocytes and fully mature MII-stage oocytes. Most of the detected changes during the GV to MI transition were related to increased levels of protein kinases, while reduced protein kinase levels and increased protein phosphorylation characterized the MI to MII transition of pig oocytes. Many of the increased protein levels associated with MI were completely or partially reversed during the MI to MII transition. The regulation of these proteins was also confirmed in maturing bovine oocytes [27].

In spite of these and other studies there is still no generally accepted list of proteins related to immature and mature oocytes in mammals, and therefore further relevant studies need to be done. It is encouraging that the reported studies identified an overlapping set of proteins that are differently expressed in immature and mature oocytes (e.g., adenylosuccinate synthetase, STIP1, Nudix, NPM2, and SPIN1). These proteins may represent the crucial proteins related to the oocyte immaturity/maturity and may be interesting for the human reproductive biomedicine in the future. Yet, discordant results between these studies might reflect the fact that oocytes were retrieved from different animal strains and that different proteomic methods were used to elucidate the proteomes. This warrants a more systematic analysis, especially targeted at human oocytes.

4.2. Proteomes of Oocytes and Surrounding Cumulus Cells.

Oocyte maturation is a highly complex process directed in part by communication between the oocyte and the surrounding follicular cells (cumulus cells). Although the nature of these interactions is largely unknown, cumulus cells are essential for proper maturation of oocytes and further embryonic development upon fertilization. Breakdown of the germinal vesicle (GV) is one of the fundamentals for development and maturation of fully grown, developmentally competent oocytes in mammals. Memili et al. [28] have analyzed the proteomes of five hundred bovine GV-stage oocytes and their surrounding cumulus cells identifying 1,092 proteins in oocytes and 4,395 proteins in the corresponding cumulus cells, 858 of which were in common. This indicates that the oocytes and surrounding cumulus cells are two distinct types of cells sharing a subset of proteins. In further steps Peddinti et al. [29] identified 811 and 1,247 proteins in bovine GV-stage oocytes and corresponding cumulus cells using differential detergent fractionation and multi-dimensional protein identification technology; 371 proteins were differentially expressed between the two cell types with statistical significance. Further modeling showed that cumulus cells, when compared to GV-stage oocytes, have higher expression levels of proteins involved in cell communication, generation of precursor metabolites and energy, and transport. The authors suggested that oocytes may depend

on the presence of cumulus cells to generate specific cellular signals to coordinate their growth and maturation [29].

4.3. Oocyte *In Vitro* Maturation at the Proteome Level. *In vitro* maturation (IVM) of oocytes is of big interest in the field of assisted reproduction and infertility treatment. Among oocytes retrieved in the *in vitro* fertilization programme there is a proportion of immature GV and MI-stage oocytes which cannot be fertilized. The possibility has been explored to aspirate these oocytes from the ovaries of women with polycystic ovary syndrome (PCOS) and mature them *in vitro* to prevent ovarian hyperstimulation in these women [30–32]. Alternatively, immature oocytes from fresh ovarian cortex tissue can be stored before chemotherapy and radiotherapy, to preserve fertility in young women with cancer and to mature and fertilize them *in vitro* [33]. In this way autotransplantation of thawed ovarian tissue for fertility restoration would be replaced by *in vitro* fertilization of *in vitro* matured oocytes from the tissue, thus avoiding the risk of retransplantation of malignant cells. In spite of this interest, oocyte *in vitro* maturation is a low-success procedure at present producing oocytes with a poor clinical outcome shown by poor embryo development and early pregnancy loss [34].

A number of proteomic studies have been performed to start understanding oocyte maturation at the molecular level. Berendt et al. [35] used 2-D DIGE saturation labeling approach for quantitative proteome profiling of bovine immature GV-stage oocytes versus *in vitro* matured MII-stage oocytes. Spots of a preparative gel from 2,200 oocytes were identified by nano-LC-MS/MS analysis, 10 of which were differentially expressed between immature and *in vitro* matured bovine oocytes including cell cycle-associated proteins and redox enzyme variants (see Table 3). Similarly, Kim et al. [36] identified proteins that were differently expressed during *in vitro* maturation of porcine oocytes using 2-DE analysis followed by mass spectrometry. They specifically focused on the proteins that were upregulated during the oocyte MII-stage when compared with the GV-stage. Similar to Berendt et al. [35] they found proteins related to the cell cycle, redox regulation (e.g., peroxiredoxin 3 (PRDX3)), and also to the cAMP-dependent pathway, which is essential for the intracellular signaling involved in oocyte maturation (see Table 3). These proteins may be involved in oocyte meiotic resumption, MII arrest, and oocyte activation. Interestingly, the results also indicated that heat shock proteins and zona pellucida glycoproteins may be involved in the oocyte *in vitro* maturation process. The protein candidates identified by these studies may help to improve the oocyte *in vitro* maturation procedure and increase the rate of *in vitro* fertilization and other ART procedures in mammals.

Interestingly, in the discussed studies there was no overlap between differentially expressed proteins in the MII-stage oocytes which matured *in vivo* or *in vitro* in comparison with immature GV-stage oocytes. This raises an important question about the potential molecular differences between *in vitro* and *in vivo* matured MII-stage oocytes which is a crucial issue that needs to be answered in the future. Of note, the expression of several epigenetics-related proteins

TABLE 3: Proteins that are differently expressed in immature and *in vitro* matured mammalian oocytes according to [35, 36].

Proteins differently expressed in immature and <i>in vitro</i> matured oocytes
Berendt et al. [35]
Ca2+-binding protein translationally controlled tumor protein (TCTP)
Enzymes of the Krebs and pentose phosphate cycles
Clusterin (CLU)
14-3-3 epsilon (YWHAE)
Elongation factor-1 gamma (EEF1G)
Polymorphic forms of GST Mu 5
Peroxiredoxin-3 (PRDX3)
Kim et al. [36]
Downregulated proteins in IVM oocytes
Similar to G patch domain and KOW motifs, partial
Heat shock 70 kDa protein 5/glucose-regulated protein (HSPA5)
Precursor (GRP78) isoform 1
TD and POZ domain-containing protein 1 (TDPOZ1)
Protein disulfide isomerase/protein disulfide-isomerase A3 precursor (ERP57)
M-phase phosphoprotein 1 (MPP1)
Chain A, Steric and Conformational Features of the aconitase mechanism
Zona pellucida sperm-binding protein 3 precursor (ZP3)
Zona pellucida glycoprotein 4 (ZP4)
Cerebellar degeneration-related protein 2 (CDR2)
Peroxiredoxin 3 (PRDX3)
Heat shock protein 27 kDa (HSP27)
Upregulated proteins
Protein kinase 5'-AMP-activated protein kinase subunit beta-1 (PRKAB1)
Myomegalin phosphodiesterase 4D interacting protein (PDE4DIP)
Major vault protein similar to lung resistance-related protein homologue (MVP)
Heat shock protein HSP 90-alpha 2 (HSP90AA2)
Heat-shock protein hsp86 (HSP86)
Heat shock protein 70.2 (HSP70.2)
Phosphoglucomutase 5 (PGM5)
Dystrobrevin alpha (DTNA)
Cytoskeletal beta actin (ACTB)
Spermine synthase (SPSY)
Galactokinase 1 (GALK1)
Transferase, HG-phosphoribosyl (HGPRT)
Glutathione S-transferase, mu 2 (GSTM2)
Glutathione-S-transferase, mu 5 (GSTM5)
Peroxiredoxin-2 (thioredoxin peroxidase 1) (PRDX2)

(e.g., NPM2, SPIN1) were differently expressed between *in vivo* matured oocytes and immature oocytes but were equally expressed in *in vitro* matured oocytes and immature oocytes.

This suggests that these proteins are differentially expressed in oocytes matured *in vitro* and *in vivo*.

5. Other Procedures of *In Vitro* Fertilization and Proteome

An important part of the *in vitro* fertilization programme is the cryopreservation of oocytes, for example, when an insufficient number of sperm is available on the day of *in vitro* fertilization [37] or for preservation of fertility before chemotherapy and radiotherapy in young women with cancer [38]. Oocytes can be preserved by two different approaches: slow freezing [39] or vitrification [40]. Proteomic studies showed that slow-freezing may affect the proteome of mouse oocytes when compared with control and vitrified mouse oocytes [41]. Similarly, Katz-Jaffe et al. [42] observed that mouse MII-stage oocytes exposed to 1.5 M propandiol (PrOH), a cryoprotectant for slow oocyte freezing, for 20 minutes exhibited significantly altered protein profiles in comparison to controls (11 proteins were downregulated and 8 were upregulated). On the other hand, they found that the temperature changes per se (during cooling to room temperature) have limited effect on the oocyte proteome. From all these data we may conclude that oocyte preservation by vitrification is less deleterious at the proteome level than standard slow-freezing procedure and may therefore be the preferred procedure. These data also indicate that proteome analysis is a powerful approach to evaluate the effect of *in vitro* fertilization procedures on the oocyte molecular status.

Moreover, a study [13] comparing mouse immature, mature, and fertilized oocytes has identified proteins that may be involved in the unique oocyte ability to reprogramme other (sperm or somatic) cell nuclei, and that thus might be very helpful in more efficient creation of induced pluripotent stem cell (iPSCs) lines in the future. The results showed that specific transcription factors and chromatin remodeling factors were more abundant in mature MII-stage oocytes than in immature oocytes. This may be crucial for the epigenetic reprogramming of sperm or somatic nuclei in oocytes.

6. Proteins Related to Epigenetics: Maternal Effect Proteins

Among a range of proteins detected by proteomics in different studies it is difficult to prioritize protein groups for functional analysis with respect to oocyte quality and maturation status, and to efficiency of *in vitro* fertilization procedures. Arguably, proteins related to epigenetic regulation represent an important class of proteins impacting on fundamental processes of early human (mammalian) development, that is, imprinting, DNA methylation, embryogenesis, and embryo development on one side and the manifestation of cancer on the other. Altered expression of these proteins may seriously affect development and embryogenesis, possibly resulting in anomalies in a baby. In Table 4 we can see the knockout mouse phenotypes related to decreased fertility, embryo arrest, and even death [43]. Several studies discussed in

this paper showed that some epigenetics-related proteins such as nucleoplasmin 2 (NPM2), spindlin 1 (SPIN1), and protein-arginine deiminase type-6 (PADI6) were differently expressed between mature and immature animal oocytes indicating that these proteins are involved in the oocyte maturation process.

The study of Zhang et al. [15] showed that proteome analysis could be a valuable resource to aid in the characterization of important maternal effect proteins in oocytes, involved in oogenesis, fertilization, early embryonic development, and revealing their molecular mechanisms of action. Using a high-performance proteomic approach they identified 625 different proteins from 2,700 mature mouse oocytes without zona pellucida. Among these proteins they also screened 76 maternal proteins (Figure 2) with high levels of mRNA expression both in oocytes and zygotes, including well-known maternal effect proteins such as MATER and NPM2. Moreover, Yurttas et al. [43] identified in mouse MII-stage oocytes a palette of abundant *bona fide* or potential maternal effect proteins, as can be seen in Table 4. In addition, the authors suggested putative “maternal effect structures” of the mouse MII-stage oocytes that they have predicted to include maternal effect proteins playing a central role in mediating the oocyte-to-embryo transition: cytoplasmic lattices, multi-vesicular aggregates, spindle apparatus, subcortical maternal complex, endoplasmic reticulum, and microtubule organizing centre.

All these findings are potentially extremely important and would need to be further researched on human oocytes for potential clinical implication. The proteins related to epigenetic regulation are definitely the first choice to evaluate the safety of assisted conception procedures at the oocyte molecular level.

7. What about Proteomics of Human Oocytes?

Although we highlighted several proteomic studies focusing on animal oocytes, to our knowledge there is no proteomic data of human oocytes in the literature at present. There are several reasons for this. Human oocytes are sensitive and scarce biological material to be researched; access to them is restricted by several ethical issues. The only potential source of oocytes is those that are discarded in daily medical practice in an *in vitro* fertilization programme, that is, immature oocytes and mature oocytes which do not fertilize after the *in vitro* fertilization procedure. In this way, oocytes can be researched after approval of the medical ethical committee and donated for a research after the donor's written informed consent. An additional issue is the relatively large number of oocytes required for proteomic studies. One possibility to get around this is by stepwise collection of oocytes, their storage in a deep-freezer or liquid nitrogen, and pooling into bigger samples. Also some issues related to cell culture would need to be avoided such as culture of oocytes in usual *in vitro* fertilization media containing high amounts of serum albumin to be able to detect a higher number of proteins

TABLE 4: *Bona fide* and putative maternal effect proteins (genes) identified in proteomic screens of mouse MII oocytes according to Yurttas et al. [43].

Maternal effect protein (gene)	Knockout mouse phenotype	Localization within oocyte	Association with maternal effect structures (MESs)
<i>Bona fide</i> maternal effect proteins			
DNA methyltransferase (cytosine-5) 1 (<i>Dnmt1</i>)	Death at E14 to E21	Cytoplasmic	Maybe CPL, MVA or SCMC
FILIA (<i>2410004A20Rik</i>)	50% decrease in female fertility	Cortex	SCMC
Factor located in oocytes permitting embryonic development—FLOPED (<i>Ooep</i>)	Two- to four-cell embryo arrest	Cortex	SCMC
Maternal antigen the embryos require—MATER (<i>Nlrp5</i>)	Two-cell embryo arrest	Cortex	SCMC
Nucleoplasmin 2 (<i>Npm2</i>)	70% decrease in female fertility, 95% of embryos arrest before blastocyst stage	Nuclear, transient cortical staining during the MII oocyte stage	Maybe SCMC
2'-5' oligoadenylate synthetase 1d—OAS1d (<i>Oas1d</i>)	30% decrease in female fertility, 40% of embryos arrest by eight-cell stage	Cytoplasm and cortex	Maybe CPL, MVA, or SCMC
Peptidylarginine deiminase 6 (<i>Padi6</i>)	Two-cell embryo arrest	Cytoplasm and cortex	CPL, maybe SCMC
Putative maternal effect proteins			
Cytosolic phospholipase A2γ (<i>Pla2g4c</i>)	NA	Cytoplasm and cortex	Spindle, maybe MVA, or SCMC
Developmental pluripotency-associated 5A—DPPA5A (<i>Dppa5a</i>)	Normal fertility due to possible redundancy with KH family members	Cytoplasm	Maybe CPL or MVA
NLR family, pyrin domain-containing 14—NLRP14 (<i>Nlrp14</i>)	NA	Cytoplasm	Maybe CPL or MVA
Spindlin (<i>Spin1</i>)	NA	Cytoplasm	Spindle, maybe CPL or MVA
Transducin-like enhancer of split 6 (<i>Tle6</i>)	NA	Cortex	SCMC

Legend: CPL: cytoplasmic lattice; SCMC: subcortical maternal complex; MVA: multivesicular aggregate; NA: not available (unknown).

by proteomics. Even under optimal conditions oocytes from the *in vitro* fertilization programme may represent only a model for natural human oocytes because they are removed from the ovarian niche, and also the surrounding cumulus cells are denuded during the procedure. In addition, nonfertilized oocytes from the programme might possess increased pathologies at the molecular level. Notwithstanding these obstacles, direct access to human oocytes is of great importance to possibly improve the outcome of *in vitro* fertilization, and therefore the elucidation of the human oocyte proteome remains an important task for the future. Ideally this includes the human oocyte secretome to gain insight in oocyte signalling and functionality.

To avoid the problems related to restricted access to oocytes, human follicular fluid has been proposed as an alternative for indirect evaluation of oocyte quality. Human follicular fluid is a complex fluid that represents the microenvironment of developing oocyte/follicle in the ovary. Follicular fluid supports oocyte maturation and ovulation; however its proteome composition is largely unknown. Potentially,

information about its protein constituents may provide a better understanding of ovarian physiology in addition to opening new avenues for investigating ovarian disorders. Follicular fluid is aspirated from the follicles during oocyte retrieval from women undergoing *in vitro* fertilization treatment and is discarded in daily medical practice, and may thus be available for proteome analysis. Indeed, following initial proteomic analyses of porcine [44] and canine [45] follicular fluid, also the proteome of human follicular fluid was reported recently [46–49], detecting up to 480 proteins [49]. The identified proteins belong to different functional categories including growth factors and hormones, receptors, enzymes, and proteins related to defense/immunity and complement activity thus reflecting a very intense cellular activity in the follicle. Interestingly, one study [46] identified potential biomarkers of good versus poor responders to ovarian stimulation in patients included in the *in vitro* fertilization programme. Moreover, they found a group of proteins (e.g., haptoglobin alpha, predominantly fetal expressed T1 domain, and apolipoprotein H) which showed an increased expression

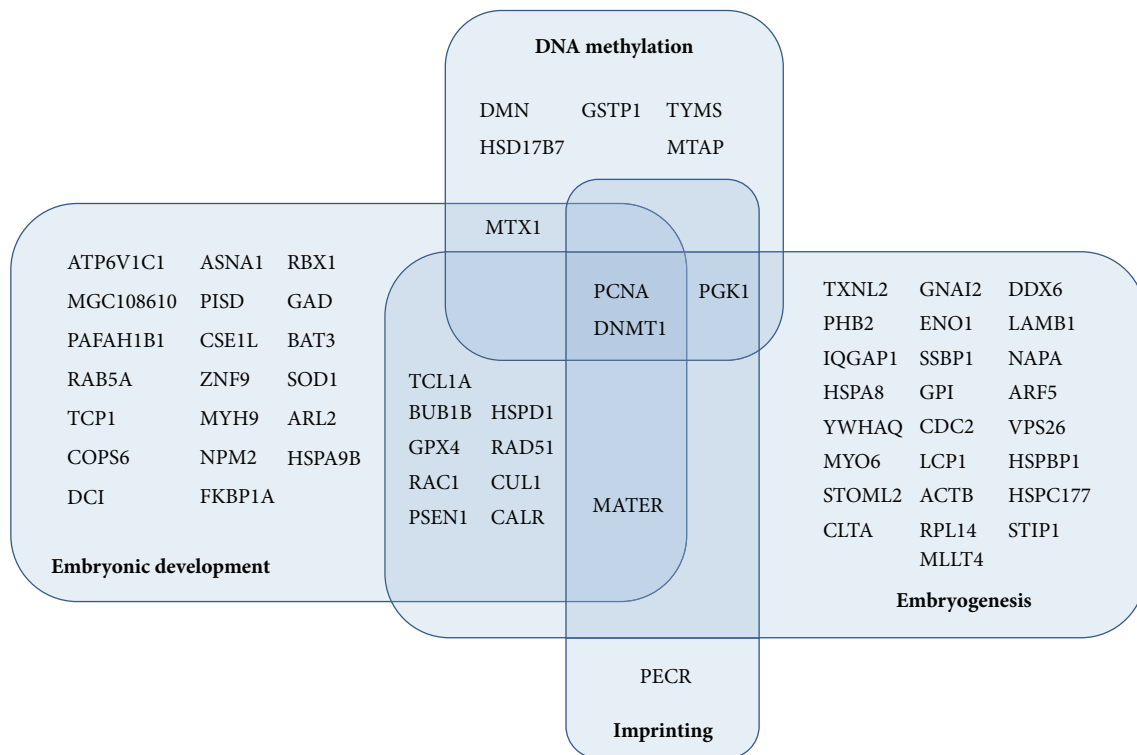


FIGURE 2: Biological processes (indicated in bold) that are regulated by maternal effect proteins in oocytes, adapted from Zhang et al. [15].

in the follicular fluid of “live birth” group of patients. Another study [48] revealed small groups of proteins related to “live birth,” “miscarriage,” and “no pregnancy.” In addition, more proteins related to biosynthesis were found in follicular fluid samples corresponding to the oocytes resulting in pregnancy after *in vitro* fertilization. Notably, there was little overlap among the proteins provided by these studies.

Although the results of these studies seem promising, further research is needed in patient groups, and proteomes need to be sampled at greater depth. Until then, the reported results need to be interpreted with caution. Clearly, and as with any clinical study, proteome analysis of follicular fluid remains a difficult task due to potential variation introduced by several factors, for example, age, ovarian pathologies and general health status, and the type of ovarian hormonal stimulation.

8. Future Perspectives of Human Oocyte Proteomics

As evidenced by the body of literature discussed above, application of proteomic technologies has made important contributions to gaining insight in proteome composition of oocytes and changes therein during development or in procedures related to assisted reproduction. This is particularly true for animal cells, mainly for reasons related to availability of cells. Although animal studies can be very insightful where many questions can be answered to uncover fundamental aspects of oocyte biology, it is clear that attention needs to be turned to human oocytes to make an impact on

natural and assisted human reproduction. One of the major bottlenecks is the scarce amount of sample that can typically be obtained, and therefore it is of crucial importance to deploy the most sensitive proteomics technologies and to keep investing in the development of such methodologies. As is apparent from the studies discussed here, 2D-gels have been the prevalent technique for proteome profiling. Because of the limitations of 2D-gels in overall sensitivity, in displaying very large or very small proteins, as well as hydrophobic proteins (including membrane proteins), they have been largely replaced by LC-based (multidimensional) peptide separation. Adoption of proteome analysis by LC-MSMS leads to higher numbers of identified proteins and requires less sample input. Importantly, implementation of miniaturized peptide separation platforms coupled to sensitive and high-resolution mass spectrometry allows for in-depth proteome profiling even for rare cell types [50]. Furthermore, accurate protein quantification is essential when aiming to identify proteins that differ in expression as a result of developmental progression, cell preservation techniques, or patient treatment. To this end, approaches using stable isotope labeling are abundant in the proteomics field, including developmental biology [51], but, with very exceptions, have not permeated in the area of oocyte biology yet. Implementation of these approaches, along with continued developments in sample preparation and mass spectrometric technologies, should enhance progress in this field. Envisioning that sample amounts in the low- μg range may suffice in the foreseeable future; a few dozen (or fewer) oocytes should be sufficient for in-depth and quantitative

proteome analysis, thus making this an accessible route for scarce human oocytes. This view may be inspired by developments in next-generation sequencing technologies now capable of sequencing genomes of single human oocytes [52], which was unimaginable just a few years ago.

9. Conclusion

With advanced proteomic technologies in place, many questions remain to be answered related to oocyte quality, maturation, and sensitivity to procedures of assisted conception. The picture that is emerging from the animal studies discussed above is that a relatively small group of proteins differ in expression between oocytes in different quality or maturation groups. Furthermore, it is encouraging that there is considerable overlap between these proteins from different studies in spite of the heterogeneity of animal species and strains, and in the methodology used. This suggests that these differences are robust, and that they may also apply to human oocytes. Maybe most interestingly, some epigenetics-related proteins were found to be differently expressed during oocyte maturation, suggesting that they deserve closer attention in the context of human reproductive medicine. We have provided some lists of additional proteins from the literature that might be of further interest to the study of human oocytes. Despite their important function in oocyte biology, secreted proteins are poorly covered in these studies, although secretome strategies are beginning to emerge [16, 53] for animal models. Extension of such studies to human oocytes could provide important cues reflecting oocyte functionality, particularly in response to different conditions. Alternatively, follicular fluid from patients who undergo *in vitro* fertilization seems to be an interesting source of informative biomolecules. We strongly believe that combined efforts between *in vitro* fertilization programmes and labs specializing in proteomics will create the conditions to have access to valuable patient material for meaningful analysis by advanced and sensitive proteomic technologies. This should provide fundamental insight into the earliest stages of human life and may come to the benefit for those who need to rely on assisted reproduction to get a baby.

Conflict of Interests

The authors declare that there is no financial or other conflict of interests related to this paper.

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Research Article

Good Preservation of Stromal Cells and No Apoptosis in Human Ovarian Tissue after Vitrification

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The aim of this study was to develop a vitrification procedure for human ovarian tissue cryopreservation in order to better preserve the ovarian tissue. Large size samples of ovarian tissue retrieved from 15 female-to-male transgender subjects (18–38 years) were vitrified using two solutions (containing propylene glycol, ethylene glycol, and sucrose at different concentrations) in an open system. Light microscopy, transmission electron microscopy, and TUNEL assay were applied to evaluate the efficiency of the vitrification protocol. After vitrification/warming, light microscopy showed oocyte nucleus with slightly thickened chromatin and irregular shape, while granulosa and stromal cells appeared well preserved. Transmission electron microscopy showed oocytes with slightly irregular nuclear shape and finely dispersed chromatin. Clear vacuoles and alterations in cellular organelles were seen in the oocyte cytoplasm. Stromal cells had a moderately dispersed chromatin and homogeneous cytoplasm with slight vacuolization. TUNEL assay revealed the lack of apoptosis induction by vitrification in all ovarian cell types. In conclusion after vitrification/warming the stromal compartment maintained morphological and ultrastructural features similar to fresh tissue, while the oocyte cytoplasm was slightly damaged. Although these data are encouraging, further studies are necessary and essential to optimize vitrification procedure.

1. Introduction

Ovarian tissue cryopreservation and its storage offer the hope to prepubertal girls or fertile age women who suffer from benign or malignant disease and want to safeguard their ovarian function against the hazardous effects of surgery, chemotherapy, and radiotherapy.

Despite the encouraging results much can still be done to minimize tissue damage during the cryopreservation procedure. It is known that the cryopreservation procedure leads to a loss in the follicular pool when compared with fresh ovarian tissue [1]. This result is principally due to ice crystal formation during the cryopreservation procedures, which can have a deleterious effect on cellular interactions and cell membranes [2].

Cryopreservation of ovarian tissue is primarily performed by slow freezing/rapid thawing for human fertility preservation and 24 live births and 4 ongoing pregnancies have been obtained after transplantation with this technique [3]. However, many studies have emphasized that the slow freezing of ovarian tissue safeguards the smallest follicles in the tissue, while it determined the greatest damage in the stroma cells [4, 5].

An emerging alternative procedure for the cryopreservation of ovarian tissue is represented by vitrification/warming. The vitrification is an ultrarapid cooling process that produces a glass-like solidification of cells by extreme elevation in viscosity, so as to avoid cellular injury caused by ice crystal formation [6–10]. In order to achieve successful vitrification,

high cooling rates, as well as high concentrations of cryoprotectants are required.

This method has been successfully applied to preserve human blastocyst and oocyte [11, 12]. For ovarian tissue, good results have been reported in rodents, domestic animals, nonhuman primates, and human; even if, data on human ovarian tissue vitrification are still limited [13].

The aim of this study was to develop a vitrification procedure for human ovarian tissue cryopreservation in order to better preserve the ovarian tissue.

2. Materials and Methods

2.1. Patients. The study was conducted in 15 female-to-male transgender subjects (FTMs), 18–38 years, (28.19 ± 5.51 , mean age \pm standard deviation) suffering from gender identity disorder and undergoing sex reassignment surgery by hysterectomy-ovariectomy (Clinical trial n°61/2007/O/Tess) at Gynecology and Pathophysiology of Human Reproduction Unit, University of Bologna, Italy. The patients have donated their ovarian tissue for research. For each patient ovarian tissue samples were analyzed at the time of sample collection (fresh tissue, t0) and after vitrification/warming (vitrified/warmed tissue, t1). t1 sample was compared with t0 sample of the same patient to minimize the interpatient variation.

2.2. Tissue Sampling. For each patient, a bioptic ovarian sample was collected at the time of surgery and immediately transferred to the laboratory in a Dulbecco's phosphate buffered solution (PBS) (Gibco, Life Technologies LTD, Paisley, Scotland) with 10% inactivated human serum (provided by the Transfusion Centre of S.Orsola-Malpighi Hospital of Bologna, Italy). Bioptic ovarian sample was cut into slices $1.5 \times 0.5 \times 0.2$ cm with a scalpel blade, placed in precooled plastic cryovials (Intermed Nunc Cryotubes, Roskilde, Denmark) and cryopreserved using the vitrification/warming protocol. For each patient, three samples (3 mm^2) were processed for light microscopy, transmission electron microscopy, and TUNEL assay (fresh tissue, t0).

2.3. Vitrification/Warming Protocol

2.3.1. Vitrification Protocol. The vitrification protocol was based on a two-step method in an open carrier. Each ovarian sample was placed in a cryovial containing 1.8 mL of equilibration solution consisting of 2 M propylene glycol (Fluka Chemical, Sigma Aldrich, SrL, Milan, Italy) + 3 M ethylene glycol (Fluka Chemical, Sigma Aldrich, SrL, Milan, Italy) + 0.2 M sucrose (Fluka Chemical, Sigma Aldrich, SrL, Milan, Italy) + 15% human serum in PBS and was transferred to a rolling system at 4°C for 30 min. Subsequently, the sample was transferred into a second cryovial containing 1.8 mL of vitrification solution consisting of 3 M propylene glycol + 5 M ethylene glycol + 0.5 M sucrose + 15% human serum in PBS and newly put onto a rolling system at 4°C for 30 min. Then, the sample was loaded in 200 μL of vitrification solution on

an open plastic support and quickly immersed into liquid nitrogen decontaminated according to Parmegiani protocol [14]. Following, the support containing the sample was placed in an empty cryovial (Intermed Nunc Cryotubes, Denmark) and stored into a liquid nitrogen tank for at least 1-2 months before warming.

2.3.2. Warming Protocol. The support containing the sample was released from the cryovial, exposed at room temperature for 30 sec, and then immersed in a warming solution consisting of 1M sucrose + 15% human serum in PBS at 39°C for 1 min, gently shaking until the ice was completely melted. Subsequently, the cryoprotectant was removed at 4°C by stepwise dilution of sucrose. The sample was transferred to the solution 1 (0.5 M sucrose + PBS + 15% human serum) for 3 min, and then to the solution 2 (0.25 M sucrose + 15% human serum in PBS) for 3 min. Finally, the warmed sample was rinsed in preequilibrated α minimal essential medium (α -MEM, Sigma Aldrich SrL; Milan, Italy) supplemented with penicillin/streptomycin (0.1 mg/mL) + 10% human serum two times at 4°C for 1 min. For each patient, warmed sample was fixed for the same analysis conducted on fresh sample (warmed tissue, t1).

2.4. Light and Transmission Electron Microscopy. For each patient, fresh and vitrified/warmed samples were fixed in a 4% paraformaldehyde solution in distilled water maintained at pH 7.4 overnight at 4°C . After osmium tetroxide postfixation and alcohol dehydration, the samples were embedded in epoxy resin (Araldite M hardener, Fluka, Buchs, Switzerland) and then sectioned using an ultramicrotome (Ultracut, Reichert, Vienna, Austria). For each sample, one 0.5 μm thick section out of every 50 was collected and stained with toluidine blue. Sections were initially observed under a microscope (Nikon) at $\times 10$ magnification to detect artifacts and discharge samples without cortical tissue. Sections were then observed at $\times 25$ to establish the development stage of the follicle, according to Gougeon classification [15], and to evaluate architecture and nuclear and cytoplasm features of stromal cells. Finally, sections were analysed at $\times 40$ to evaluate follicle and interstitial injury on at least 10 random microscopic high power fields (HPF) and scored using the method previously described by Fabbri et al. [5]. Semithin sections were viewed in a blind fashion by two different pathologists using a Leitz Diaplan light microscope equipped with a CCD JVC video camera, and digitized images were analyzed with Image ProPlus software.

After semithin sectioning, the 60 nm thick sections were collected on 200 mesh grids, stained with uranyl acetate followed by lead citrate and viewed using a Philips 410 T transmission electron microscope at 80 kV, in order to evaluate the ultrastructural features of follicles before and after vitrification/warming. Chromatin pattern, integrity of organelles, and membranes were carefully screened for oocytes, granulosa as well as stromal cells, according to previously reported subcellular criteria [5].

TABLE 1: Number and % of degenerated follicles on the total number of follicles parted for primordial, primary, secondary, preantral, and antral stage.

Samples (n = 15)	Degenerated follicles/total number of follicles (%)					TOT.
	Primordial/intermediary	Primary	Secondary	Preantral	Antral	
t0	215/739 (29)	4/15 (27)	1/2 (50)	0/1 (0%)	0/0 (0%)	757
t1	221/690 (32)	9/28 (32)	1/3 (33)	0/0 (0%)	0/1 (0%)	722

t0: fresh tissue; t1: vitrified/warmed tissue.

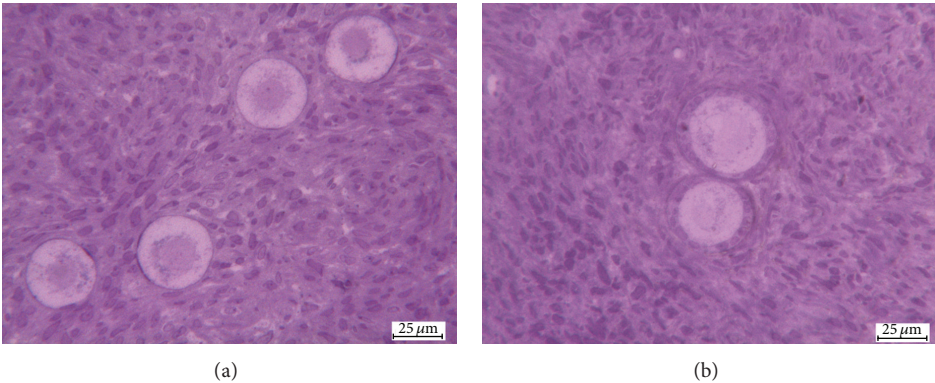


FIGURE 1: Light microscopy images of t0 (a) and t1 (b) samples. Well-preserved clusters of follicles displaying euchromatic nuclei and preserved oocyte cytoplasm. Compact stromal cells. t0 = fresh tissue and t1 = vitrified/warmed tissue.

2.5. Apoptosis Analysis. For each patient, fresh and vitrified/warmed samples were fixed in 4% formaldehyde, embedded in paraffin, and serially sectioned at a thickness of 4 μm . Every 5 slides, one was stained with haematoxylin and eosin (Merck KGaA, Darmstadt, Germany) for histological evaluation; the other slides were TUNEL stained, for apoptosis evaluation (POD, Roche, Mannheim, Germany), according to the manufacturer’s instructions. Brown nuclei indicated TUNEL-positive cells. Follicles with more than two TUNEL-positive granulosa cells were considered to be apoptotic, as well as those follicles with TUNEL-positive oocytes. A total of 100 follicles were analyzed for each experimental condition. The number of apoptotic stromal cells was analyzed in a 100 μm^2 square (randomly assigned in the sections) and was counted three times and then averaged. The percentage of apoptotic cells was calculated as follows: apoptotic cell number/total cell number \times 100 and quantified in a double blind fashion using a Leitz Diaplan light microscope equipped with a CCD JVC video camera. Digitized images were analyzed with Image ProPlus software.

2.6. Statistical Analysis. For each variable (follicle damage and interstitial edema), at least 10 fields were scored on an integer scale of 0–3. Differences in the mean score among fresh and vitrified/warmed samples were statistically analyzed using Kruskal-Wallis test followed by Dunn multiple comparison test (GraphPad Prism version 4). The Student’s *t*-test was used to analyze the percentage of apoptotic cells observed in fresh and vitrified/warmed samples (GraphPad Prism version 4). *P* < 0.05 was considered statistically significant.

3. Results

3.1. Light and Transmission Electron Microscopy. A total of 1479 follicles (from fresh and vitrified/warmed samples) were analyzed by light microscopy to evaluate the morphology, the developmental stage, and the percentage of degenerated follicles on the total number of follicles (Table 1). The number of follicles was equally distributed among fresh and vitrified/warmed samples (53% versus 47%; *P* = 0.905). The follicle density (follicle number per mm^2 of the overall section area) was $4.28 \pm 6.15/\text{mm}^2$ in fresh and $4.12 \pm 6.48/\text{mm}^2$ in vitrified/warmed tissue (*P* = 0.269). Overall, 1429 follicles (96.5%) were primordial and intermediary, 43 (3%) primary, 5 (0.36%) secondary, 1 (0.07%) preantral, and 1 (0.07%) antral.

In fresh samples follicles showed close adherence between oocyte and granulosa cells with normal morphological appearance: the oocytes had many mitochondria located around the nucleus, homogenous cytoplasm, and no vacuoles were observed. Stromal cells had oval- to spindle-shaped nucleus with a finely dispersed chromatin, small cytoplasm vacuoles were seen, and no interstitial edema was present (Figure 1(a)).

The vitrified samples showed oocyte nucleus with slightly thickened chromatin and irregular shape, while granulosa cells were well preserved. The architecture of the ovarian stromal was intact, maintaining the features of not vitrified tissue even though an increased cytoplasm vacuolization was observed (Figure 1(b)).

The data obtained by statistical analysis showed that no significant differences were observed in the overall preservation of ovarian tissue processed according to the vitrification protocol when compared with fresh tissue (Figure 2).

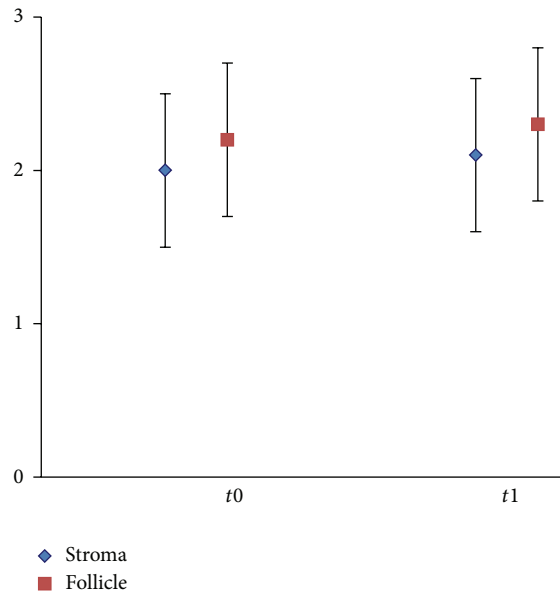


FIGURE 2: Statistical analysis, showing scores for follicle and stroma preservation in fresh (t0) and vitrified/warmed (t1) ovarian cortical tissue. Values are expressed as mean \pm SD. Score interpretation: 0 = good preservation, 1 = poor preservation, 2 = bad preservation, and 3 = worst preservation. $P = 0.17$ for stroma and $P = 0.53$ for follicles.

To extend and validate LM evaluation, ultrastructural analysis was performed. A total of 132 follicles, equally distributed among fresh and vitrified/warmed samples (70 versus 62, $P = 0.712$), were analyzed. The fresh samples showed follicles containing large oocytes with regular nuclei, finely dispersed chromatin, nuclear pores, and abundant cytoplasm. Mitochondria appeared typically rounded, with a low-density matrix and few peripheral cristae, clustered in clouds or arranged in a rosette-like pattern around a dense, amorphous or finely granular intermitochondrial substance. Golgi apparatus was well developed; a few cisternae of rough endoplasmic reticulum were seen and occasional lipid inclusions and lipofuscin bodies as well. A close adhesion between oocyte and follicular cells was commonly seen. Granulosa cells showed flat nuclei with one or two reticular nucleoli. Rod-shaped mitochondria, free ribosomes, rough and smooth endoplasmic reticulum, and scattered vacuoles were observed in the cell cytoplasm (Figure 3(a)). The stroma showed spindle cell with moderately dispersed chromatin and homogeneous cytoplasm and slight signs of vacuolization (Figure 3(b)).

The vitrified/warmed follicles had nucleus with slight irregular shape and finely dispersed chromatin. Clear vacuoles were seen in the oocyte cytoplasm and a slight clarification of the cytoplasmic matrix as well. Cytoplasmic organelles were altered in shape and distribution. Irregularly shaped or swollen mitochondria were found scattered in the cytoplasm or around large vacuoles. Granulosa cells were adherent to oocyte (Figure 3(c)). Stromal cells showed moderately dispersed chromatin and homogeneous cytoplasm with slight vacuolization and enlarged mitochondria (Figure 3(d)).

3.2. TUNEL Assay. Only follicles with a visible oocyte nucleus were analyzed. The percentage of apoptotic follicles was 5% and 6% in fresh and vitrified/warmed samples, respectively ($P = 0.13$). The percentage of DNA damaged stromal cells evaluated in fresh and vitrified/warmed samples was 11% and 12%, respectively ($P = 0.18$; Figure 4).

4. Discussion

Today the cryopreservation of ovarian tissue before the start of cancer treatments is proposed as an option for preserving fertility. It allows recovering of a large number of follicles; it may be performed at any time of the menstrual cycle avoiding delay in the onset of antineoplastic therapy; it is particularly indicated in patients with hormone-related cancers, and it is the only option for prepubertal patients [3].

The critical points in the cryopreservation of ovarian tissue are determined by the presence of heterogeneous cellular components (oocytes, granulosa, and stromal cells) with different properties, which make the osmotic dehydration and rehydration extremely variable [16].

Cryopreservation strategies for ovarian tissue have therefore aimed to optimize methods and protocols for the preservation of whole tissue [13].

In the last decade there has been a great interest for the vitrification, since it might prevent tissue damage due to ice crystal formation. The success of vitrification depends on many factors such as sample size, type and concentration of cryoprotectants, temperature of exposure to vitrification solution, stepwise addition of vitrification solution, carrier system, quality of samples, and technical expertise [13].

Because of so many variables, the results obtained from vitrification are discrepant from species to species and within the same species [13].

The vitrification of ovarian tissue in animal models (mouse, sheep, ovine, bovine, monkey, hamster, rabbit, and baboon) has produced promising results: follicles maintain the same survival rates, morphology, and ultrastructure comparable to fresh follicles, yielding even live births in some species [3, 13].

In human, many studies support vitrification as the method of choice for ovarian tissue cryopreservation, providing similar results to conventional freezing, with the additional advantage of preserving the ultrastructure of stromal tissue that is usually affected by freezing [1, 2, 13, 17–19]. However the live birth of babies from transplantation of vitrified ovarian tissue has not yet been obtained. Therefore, despite the encouraging results, absolute conclusions cannot easily be drawn.

In our study large size samples and two solutions were used to vitrify human ovarian tissue in an open system. In order to promote the dehydration of large size samples, a combination of ethylene glycol, propylene glycol, and sucrose at different concentrations and a long equilibration time were applied. Light and transmission electron microscopy and TUNEL analysis were applied to monitor the efficiency of our vitrification protocol.

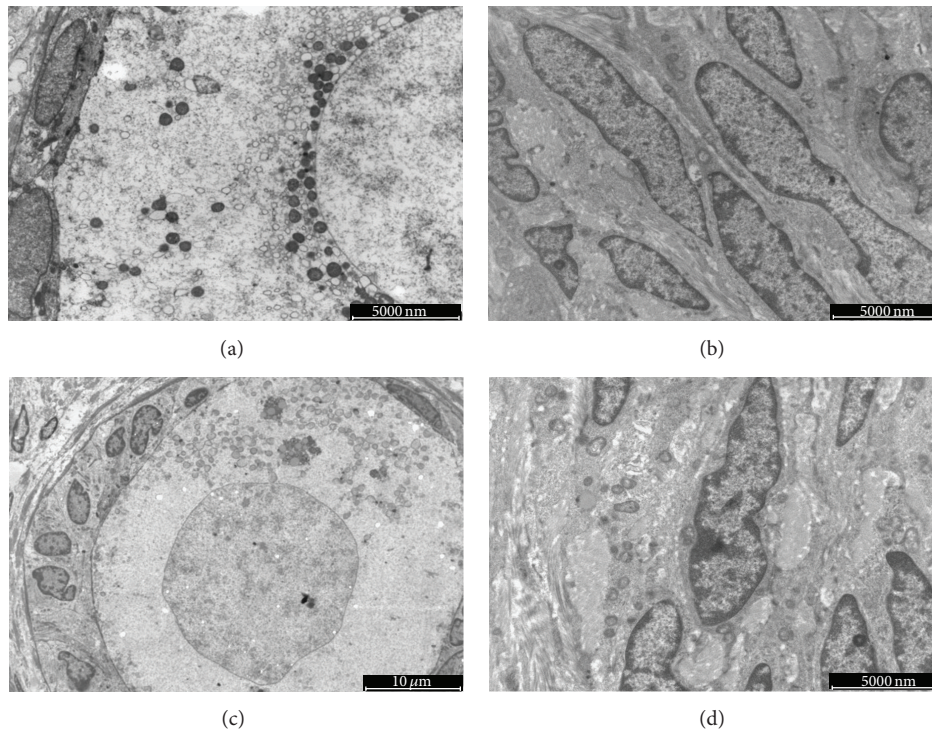


FIGURE 3: Ultrastructure images of t0 ((a), (b)) and t1 ((c), (d)) ovarian cortical tissue. A primordial follicle with a layer of flattened granulosa cells. The oocyte nucleus shows homogeneous euchromatin surrounded by the nuclear membrane. Cytoplasm is well organized with perinuclear round mitochondria (a). Spindle stromal cells with moderately dispersed chromatin and homogeneous cytoplasm (b). A primary follicle with a layer of cuboidal granulosa cell firmly attached to the oocyte. The oocyte nucleus has a slight irregular outline and euchromatin content. Mitochondria are eccentrically placed at one side of the oocyte cytoplasm (c). Stromal cells have moderately dispersed chromatin, compact cytoplasm with enlarged mitochondria (d). t0 = fresh tissue and t1 = vitrified/warmed tissue.

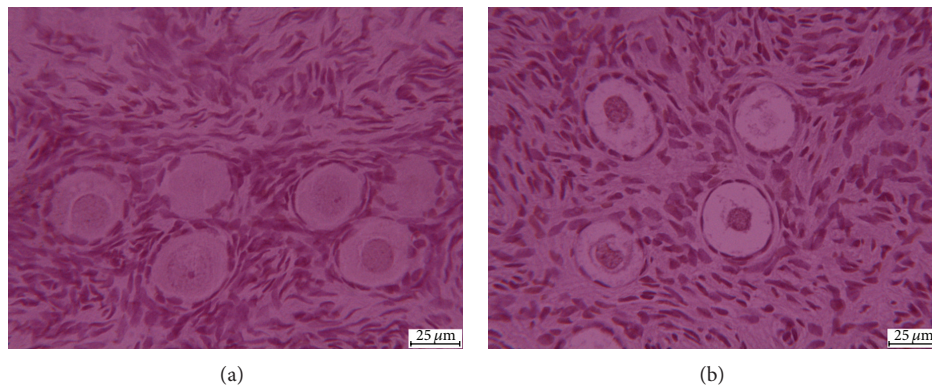


FIGURE 4: TUNEL images of t0 (a) and t1 (b) samples. t0 = fresh tissue and t1 = vitrified/warmed tissue.

The literature data show that the sample's sizes have a strong influence on the preservation; in fact using small specimens with better results has been obtained [13]. However studies in sheep by Bordes group [20] obtained the birth of four lambs and the recovery of ovarian endocrine function by half ovary vitrification [20, 21]. In addition, data reported by Ferreira et al. [22] on bovine ovarian cortex showed that there was an increased risk of morphological damage to primary and primordial follicles when the tissue slices were cut with all dimension larger than 2 mm, provided that one of the

dimensions of the fragment slice is maintained ≤ 2 mm [22]. The vitrification of large size samples of human ovarian tissue could be particularly useful, because the handling of the tissue and the gain in terms of follicular population are better using the larger samples [23].

Our results show that after vitrification/warming the stromal compartment maintains morphological and ultrastructural features similar to those seen in fresh tissue. Regarding follicles, the granulosa cells appear well preserved, while the oocyte appears slightly negatively affected by vitrification,

as shown by increased cytoplasm vacuolization and organelle changes. These features might be considered as early signs of follicular degeneration, likely due to an inadequate dehydration and/or osmotic stress [24].

Our results are in agreement with those reported by other authors [25–28]. Gandolfi et al. [25] showed that vitrification caused extensive morphological damage to primordial follicle in human ovarian tissue. Isachenko et al. [26] compared conventional freezing and vitrification of human ovarian tissue in terms of follicular quality, steroidogenic activity, and proliferative potential by GAPDH gene expression. The results provided evidence that conventional freezing allowed a better preservation of all types of follicles and a higher proliferative capacity of the tissue than vitrification, concluding that conventional freezing is the method of choice for the cryopreservation of human ovarian tissue. Irregularly shaped or swollen mitochondria scattered in the cytoplasm of both oocytes and follicular cells were reported by Zhou et al. [27] using conventional vitrification. Oktem et al. [28] reported that vitrified ovaries contained statistically significantly fewer primordial follicles and reported statistically significantly less hormonal production in vitro when compared with fresh and slow-frozen ovaries.

In the present study we also evaluated the frequency of apoptosis in ovarian tissue after vitrification. In agreement with previous studies, TUNEL assay does not show evidence of any statistical difference in apoptosis between fresh and vitrified samples [17, 18]. This finding suggests that the cryodamages induced by vitrification occur mainly in cytoplasmic organelles and cell membranes, preserving the nuclear compartment. In fact, at nuclear level the most likely consequence of vitrification would be the necrosis process, which can be better demonstrated using careful morphological and ultrastructural analysis [16].

Certainly a limitation of the present study has been the lack of functional tests that allowed determining if the damage done to oocytes was reversible and transitory or not. Further investigation such as xeno-transplantation might give information about viability of the follicle within the tissue. Nevertheless, tissue culture might be an excellent alternative method to demonstrate the viability of the tissue.

5. Conclusions

In conclusion, our vitrification protocol showed a good preservation of the stromal compartment, as also reported by other authors in the literature. This finding indicates that vitrification protocols are efficient for human ovarian tissue cryopreservation compared with conventional freezing, because stromal damages are avoided. However, in our study slight damages to the oocytes were observed after vitrification. Therefore, further studies are necessary and essential to optimize the vitrification protocol and to decide to switch from conventional freezing to vitrification for human ovarian tissue cryopreservation. The final proof to propose vitrification of ovarian tissue to patients will only be the birth of healthy babies after reimplantation of vitrified tissue.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Review of Clinical Trials on Effects of Oral Antioxidants on Basic Semen and Other Parameters in Idiopathic Oligoasthenoteratozoospermia

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Infertility affects 50 to 80 million people worldwide. Male factor is a cause of infertility in almost half of cases, mainly due to oligoasthenoteratozoospermia (OAT). With common diagnostic methods no cause can be found in approximately 30% of cases of male infertility due to OAT and these are considered idiopathic. Reactive oxygen species (ROS) play an important role in male infertility and are proved to be higher in infertile men; antioxidants could oppose their effect. The aim of this paper was to review the literature on clinical trials in the period from year 2000 to year 2013 studying the effects of various types of antioxidant supplements on basic and other sperm parameters and pregnancy rates in subfertile males with idiopathic OAT. The majority of studies were randomized and placebo controlled and confirmed beneficial effect of antioxidants on at least one of the semen parameters; the biggest effect was determined on sperm motility. In many of these trials combinations of more antioxidants were assessed. The optimal dosages of one or more antioxidants were not defined. We concluded that antioxidants play an important role in protecting semen from ROS and can improve basic sperm parameters in case of idiopathic OAT.

1. Introduction

Almost 15% of all couples trying to conceive are affected by infertility, and in almost half of these cases male infertility is the sole or a contributing factor [1]. While conditions such as varicocele, cryptorchidism, and hypogonadism are definable causes for infertility, no cause may be determined for an abnormal semen analysis in over 25% of cases [2]. Such idiopathic infertility and oligoasthenoteratospermia (iOATs) is a condition in which sperm concentration, the proportion of motile sperms, and the proportion of morphologically normal sperms are below the World Health Organization (WHO) reference values [3].

Elevated reactive oxygen species (ROS) levels in the semen may be an etiologic factor for male infertility [4]. It is estimated that 25% of infertile men possess high levels of semen ROS, whereas fertile men do not have high levels of semen ROS [5, 6]. ROS are needed for capacitation, the acrosome reaction, and ultimately fertilization [7]. However,

their uncontrolled production is detrimental to cell function as they damage a variety of biomolecules such as lipids, amino acids, carbohydrates, protein, and DNA and adversely affect sperm function [8] due to DNA damage [9, 10], reduced motility [11], and defective membrane integrity [12, 13]. Spermatozoa are particularly susceptible to oxidative injury due to the abundance of plasma membrane polyunsaturated fatty acids. These unsaturated fatty acids provide fluidity that is necessary for membrane fusion events (e.g., the acrosome reaction and sperm-egg interaction) and for sperm motility [14]. The human ejaculate contains a number of potential sources of ROS. These include leukocytes, germ cells, or abnormal sperms [15]. At the same time, a number of cellular molecules called antioxidants, which protect the cell from excessive ROS-induced lipid peroxidation, are also present within the ejaculate [16]. Studies have shown that seminal antioxidant capacity is suppressed in infertile men with high ROS levels compared to men with normal levels of ROS [17, 18].

2. Materials and Methods

We searched PubMed with keywords, including combinations of search terms such as “male infertility” and “antioxidants.” We searched for reviews, controlled and randomized controlled clinical studies. From the numerous search results for the period between 1st January 2000 and 31st December 2013, 32 primary studies on idiopathic oligoasthenozoospermia (OAT) were chosen and their data were gathered in order to provide a complete overview of the literature. Given the different antioxidants used (both alone and in combination), the different dosages, different duration of treatment, and various number of participants (from very small groups to large researches), we looked up for statistical significance of changes in basic sperm parameters and pregnancy rates.

3. Results and Discussion

The review of the studies on antioxidants in clinical studies is illustrated in Table 1.

3.1. Sperm Concentration. Low sperm concentration or oligozoospermia is defined as concentration less than 15×10^6 spermatozoa/mL according to WHO reference value from 2010 [51] and less than 20×10^6 spermatozoa/mL according to WHO reference values from 1999 [52], which were considered in most of researches in this review. Many researches showed significant improvements in sperm concentration after oral intake of different antioxidants [19–31]. Most of these researches investigated combination of different antioxidants, like L-carnitine, coenzyme Q10 (CoQ10), vitamin C, vitamin E, zinc (Zn), selenium (Se), and so forth. But there are also some studies that investigated only one type of antioxidant. Safarinejad et al. showed that intake of 200 mg CoQ10 daily for 26 weeks improved sperm concentration in study group ($28.7 \pm 4.6 \times 10^6$ spermatozoa/mL) versus placebo group ($16.8 \pm 4.4 \times 10^6$ spermatozoa/mL) ($P = 0.005$) [23]. After 6 months of intake of combination of 25 mg clomiphene citrate and 400 mg vitamin E per day sperm concentration improved from $10.2 \times 10^6 \pm 4.14$ spermatozoa/mL to $18 \times 10^6 \pm 15$ spermatozoa/mL ($P = 0.0025$) [26]. There was also significant improvement in sperm concentration from $14.3 \pm 7.38 \times 10^6$ spermatozoa/mL to $32.8 \pm 10.3 \times 10^6$ spermatozoa/mL ($P < 0.001$) after consumption of 1 g of vitamin C twice daily taken for 2 months as proved by Akmal et al. [28].

3.2. Sperm Motility. Asthenozoospermia is defined as less than 40% of motile spermatozoa [51] and according to WHO reference value from 1999 less than 50% of motile spermatozoa [52]. 20 out of 32 studies in our review proved significant improvement in sperm motility after the use of antioxidants [19, 20, 22–39]. Improvement in sperm motility has been shown mostly in researches considering mixture of more antioxidants such as selenium and vitamin E [38, 39]. Most of studies with just one type of antioxidant were about CoQ10 but in different dosages and in different duration of consuming [22–24, 37]. Kumar et al. showed

that consumption of herbal-mineral supplement Addyzoa for 3 months improved total and progressive sperm motility in study group. Total motility improved from $23.2 \pm 17.3\%$ before the treatment to $33.4 \pm 23.2\%$ after the treatment ($P = 0.008$). Progressive motility improved from $15.7 \pm 12.6\%$ before treatment to $22.6 \pm 18.0\%$ after treatment with Addyzoa ($P = 0.024$) [33]. Wang et al. showed that L-carnitine in combination with vitamin E taken for 3 months significantly improved forward sperm motility from $28.6\% \pm 9.2\%$ to $45.4\% \pm 11.1\%$ ($P < 0.01$), compared with just vitamin E [35]. After treatment with 200 mg CoQ10 twice daily for 6 months sperm motility improved from $9.13\% \pm 2.50\%$ before the therapy to $16.34\% \pm 3.43\%$ after the therapy ($P < 0.05$) [37].

3.3. Sperm Morphology. WHO reference values from 1999 [52] defined teratozoospermia as less than 14% of normal shape and form spermatozoa according to strict Krüger criteria. Although WHO reference values from 2010 define teratozoospermia as less than 4% of normal shape and form spermatozoa [51] strict Krüger criteria are still used as reference value for assessing sperm morphology. L-carnitine in combination with CoQ10, vitamins E and C, zinc, selenium [20, 40], CoQ10 alone [23, 24], pentoxifylline [25], N-acetylcysteine with Se [27], vitamin C alone [28], combination of papaya, beta-glucan, lactoferrin, vitamins C and E [36], Se, and vitamin E [38], and pycnogenol [41] significantly improved sperm morphology. Therapy with 200 mg CoQ10 daily for 26 weeks improved sperm morphology in 114 participants in study group to $17.6\% \pm 4.4\%$ versus $14.8\% \pm 4.1\%$ in 114 participants in placebo group ($P = 0.01$) [23]. Safarinejad also showed that intake of 400 mg of pentoxifylline twice daily for 24 weeks of treatment phase significantly improved percentage of sperm with normal morphology to $25.4 \pm 4.3\%$ in study group versus $17.4 \pm 4.2\%$ in placebo group ($P = 0.001$) [25]. Combination of 20 mg beta-glucan, 50 mg fermented papaya, 97 mg lactoferrin, 30 mg vitamin C, and 5 mg vitamin E, twice per day for 3 months, improved percentage of morphologically normal sperm in 36 participants from $17.0 \pm 5.2\%$ to $29.8 \pm 6.5\%$ ($P < 0.01$) [36].

3.4. Sperm DNA Fragmentation and Chromatin Integrity. ROS can cause sperm DNA damage and integrity of sperm DNA can be measured with DNA fragmentation. The levels of sperm-derived ROS (measured in sperm preparations having minimal leukocyte contamination) have been associated with sperm DNA damage [53]. High level of denatured DNA in spermatozoa with large nuclear vacuole could arise from precocious decondensation and disaggregation of sperm chromatin fibers [54]. Dietary antioxidants may be beneficial in reducing sperm DNA damage, particularly, in men with high levels of DNA fragmentation [5]. Five out of 32 studies confirmed that the usage of different antioxidants had important influence on DNA fragmentation and chromatin integrity [20, 42–46]. Song et al. showed that combination of Chinese medicine Compound Xuanju Capsule with vitamin E taken for 3 months decreased degree of DNA fragmentation index (DFI) after therapy to 29.57 ± 12.19 compared just to

TABLE 1: Study characteristics and the effect of oral antioxidants on basic and other semen parameters.

Study/author	Year	Patients/test	Number of patients	Antioxidant/duration of th.	Significant improvement	Nonsignificant improvement	Negative effect
Wirlitner et al. [19]	2012	OAT versus non-OAT, MSOME	147	Fertilovit Mplus/2-12 months	↑ concentration and motility of sperm	Morphology	
Abad et al. [20]	2013	AT/DFI, basic sperm parameters	20	L-Carnitine 1500 mg; vitamin C 60 mg; CoQ10 20 mg; vitamin E 10 mg; Zn 10 mg; vitamin B9 200 µg; Se 50 µg; vitamin B1 21 µg/3 months	DNA integrity ($P < 0.01$), the proportion of DDS ↓ ($P < 0.05$). ↑ in concentration, motility, vitality, and morphology parameters.		
Safarinejad [21]	2011	iOAT	238 (analysis on 211) SG: 106 PG: 105	SG: eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), 1.84 g per day versus PG/32 weeks	SG: ↑ of sperm cell total count (from $38.7 \pm 8.7 \times 10^6$ to $61.7 \pm 11.2 \times 10^6$, $P = 0.001$) and sperm cell concentration (from $15.6 \pm 4.1 \times 10^6$ /mL to $28.7 \pm 4.4 \times 10^6$ /mL, $P = 0.001$). Seminal plasma EPA and DHA conc. were positively correlated with seminal plasma SOD-like and catalase-like activity (both $P = 0.001$).	In seminal plasma, both SOD-like and catalase-like activity were positively correlated with sperm count, sperm motility, and sperm morphology.	
Safarinejad [22]	2009	iOAT/semen analyses, AR, immunobead test for antisperm antibody, and determination of resting levels of LH, FSH, prolactin, testosterone, and inhibin B	212 (SG: 106, versus PG: 106)	CoQ10 300 mg/26 weeks followed by a 30-week treatment-free phase	SG: ↑ in sperm density and motility (each $P = 0.01$). ↓ FSH and LH at the 26-week treatment phase (each $P = 0.03$). By the end of the treatment phase the mean AR had increased from $14\% \pm 8\%$ and $15\% \pm 8\%$ to $31\% \pm 11\%$ and $16\% \pm 10\%$ in the CoQ10 and placebo groups, respectively ($P = 0.01$).		
Safarinejad et al. [23]	2012	iOAT/semen parameters, seminal plasma TAC, FSH, and inhibin B	228 SG: 114 PG: 114	CoQ10 200 mg/day/26 weeks	SG: ↑ in sperm density ($28.7 \pm 4.6 \times 10^6$ /mL versus $16.8 \pm 4.4 \times 10^6$ /mL ($P = 0.005$)), sperm motility ($35.8\% \pm 2.7\%$ versus $25.4\% \pm 2.1\%$ ($P = 0.008$)), and sperm morphology ($17.6\% \pm 4.4\%$ versus $14.8\% \pm 4.1\%$ ($P = 0.01$)). FSH ↓ ($P = 0.02$), inhibin B ↑ ($P = 0.01$)		

TABLE 1: Continued.

Study/author	Year	Patients/test	Number of patients	Antioxidant/duration of th.	Significant improvement	Nonsignificant improvement	Negative effect
Safarinejad [24]	2012	iOAT/semen parameters and pregnancy rates	287	CoQ10 300 mg orally twice daily/12 months	Mean sperm conc., sperm progressive motility, and sperm with normal morphology improved by 113.7, 104.8, and 78.9%, respectively (all $P < 0.05$).	The overall spontaneous pregnancy rate was 34.1% within a mean of 8.4 ± 4.7 months.	
Safarinejad [25]	2011	iOAT/semen parameters, testosterone, LH, FSH, and inhibin B, seminal plasma SOD-like activity, and acrosome reaction	254 (SG: 127; PG: 127)	SG: PTX (pentoxyfylline) 400 mg twice daily/4-week screening phase, a 24-week treatment phase, and a 12-week treatment-free period	SG after PTX: ↑ sperm conc. (mean value, from $26.4 \pm 4.6 \times 10^6$ /mL to $16.2 \pm 3.4 \times 10^6$ /mL), sperm motility (mean value, from $35.8 \pm 4.2\%$ to $26.4 \pm 2.4\%$), and sperm with normal morphology (mean value, from $25.4 \pm 4.3\%$ to $17.4 \pm 4.2\%$) (all $P = 0.001$); mean SOD-like and catalase-like activity ↑ than in the semen of PG (46.4 ± 2.4 versus 36.3 ± 1.3 U/mL and 371 ± 44 versus 301 ± 14 U/mL, respectively, both $P = 0.003$). The AR was observed to be ↑ in PTX group ($P = 0.01$).		
Ghanem et al. [26]	2010	iOA/basic semen parameters, pregnancy incidence	SG: 30 PG: 30	Clomiphene citrate 25 mg/day + vit. E 400 mg/day/6 months	SG: sperm conc.: $10.2 \times 10^6 \pm 4.14 \rightarrow 18 \times 10^6 \pm 15$ ($P = 0.0025$); progressive motility: $4\% \pm 6 \rightarrow 7\% \pm 10$ ($P = 0.0286$). Spontaneous pregnancy incidence, SG: 36.7%, versus PG: 13.3% ($P = 0.037$)		
M. R. Safarinejad and S. Safarinejad [27]	2009	iOAT/serum T estradiol, FSH, LH, prolactin, inhibin B, Se, and N-acetyl-cysteine. Semen analysis, seminal plasma Se, and N-acetyl-cysteine.	468 SG 1: 116 SG 2: 118 SG 3: 116 PG: 118	SG 1: Se 200 µg/day SG 2: N-acetyl-cysteine 600 mg/day SG 3: Se 200 µg + N-acetyl-cys 600 mg/day/26 weeks + 30-week treatment-free period	A strong correlation was observed between the sum of the Se and N-acetyl-cysteine concentration and mean sperm concentration ($r = 0.67$, $P = 0.01$), sperm motility ($r = 0.64$, $P = 0.01$), and percent normal morphology ($r = 0.66$, $P = 0.01$).	Se + N-ac-cy: ↓ FSH, ↑ T, inhibin B	

TABLE 1: Continued.

Study/author	Year	Patients/test	Number of patients	Antioxidant/duration of th.	Significant improvement	Nonsignificant improvement	Negative effect
Akmal et al. [28]	2006	O/semen parameters	13	vitamin C 1 g twice daily/2 months	Mean sperm count: $14.3 \pm 7.38 \times 10^6$ sperms/mL to $32.8 \pm 10.3 \times 10^6$ sperms/mL ($P < 0.001$), mean sperm motility: $31.2 \pm 9.61\%$ to $60.1 \pm 8.47\%$ ($P < 0.001$), and mean sperms with normal morphology: $43 \pm 7.87\%$ to $66.7 \pm 4.77\%$ ($P < 0.001$).		
Shi et al. [29]	2004	OA/semenal routine analysis	34	Xinxiabao (Zn and Se tablets) three times a day/90 days + five tablets at a time for 90 days in succession	The sperm quality was improved 60 days and 90 days after treatment. 5 cases (14.7%) showed remarkable effect, 25 (73.5%) improved.	4 cases (11.8%) did not respond.	
Suzuki et al. [30]	2003	O and A versus normozoospermia/sperm parameters, serum hormones, and SOD activity in the serum and the seminal plasma + the testicular artery	SG: 47 CG: 16	Sairei-to 9 g/day/3 months	SG: total sperm conc. (17.1 ± 20.0 to $28.7 \pm 35.5 \times 10^6$ /mL, $P = 0.02$) and sperm motility ($30.1\% \pm 21.6$ to $45.8\% \pm 24.4$, $P < 0.0001$) and the pulsatility index of the testicular artery \downarrow (2.03 ± 0.84 to 1.64 ± 0.48 , $P = 0.04$) 20 patients (66%): \uparrow sperm conc., 16 (53%) \uparrow motility. The median change in concentration was 22 million/mL, motility 25%. Higher baseline concentrations (more than 5 million/mL) were associated with significant improvement and resulted in six spontaneous pregnancies in 26 patients (23%).	After th. serum hormones and SOD activity did not change significantly in either group. CG: no significant change in sperm conditions or testicular artery flow.	
Gupta and Kumar [31]	2002	Idiopathic nonobstructive O/A/T spermia/semen analysis	30	Lycopene 2000 mcg, twice a day/3 months		14 patients (46%) \uparrow in sperm morphology (median change 10%). Baseline sperm concentration less than 5 million/mL was associated with no significant improvement.	
Busetto et al. [32]	2012	Idiopathic AT/basic sperm parameters	114 (96 finished)	L-Carnitine 145 mg, acetyl-L-carnitine 64 mg, fructose 250 mg, citric acid 50 mg, Se 50 μ g, CoQ10 20 mg, Zn 10 mg, ascorbic acid 90 mg, cyanocobalamin 1.5 μ g, and folic acid 200 mcg once a day/4 months	\uparrow Mean sperm progressive motility: 18.3 ± 3.8 to 42.1 ± 5.5 , 16 patients achieved pregnancy during the study.	Concentration and morphology	

TABLE 1: Continued.

Study/author	Year	Patients/test	Number of patients	Antioxidant/duration of th.	Significant improvement	Nonsignificant improvement	Negative effect
Kumar et al. [33]	2011	At least one parameter of OAT/basic semen parameters, ROS, TAC, and DFI (SCSA)	SG: 21 PG: 23	herbal-mineral supplement Addyzoa/3 months	SG: total motility: 23.2 ± 17.3% → 33.4 ± 23.2% (<i>P</i> = 0.008) Progressive motility: 15.7 ± 12.6% → 22.6 ± 18.0% (<i>P</i> = 0.024)		
Chen et al. [34]	2012	O, A/sperm concentration and % of progressively motile sperm, the rate of clinical pregnancy	Oligosp: 64 (SG: 33 + CG: 31) Asthenozo: 42 (SG: 22 + CG: 20)	Oligospermia: CG: tamoxifen 10 mg bid + vit. E 100 mg tid Asthenospermia: CG: levocarnitine oral solution 1 bottle bid SG: levocarnitine oral solution 1 bottle bid + vit. E 100 mg tid/3 months	the number of spontaneous pregnancies after th. were CG: 0, and SG: 6 (<i>P</i> < 0.01). Asthenospermia: after th. the numbers of cases evaluated as with no or slight improvement in the % of progressively motile sperm were 7 and 2 (<i>P</i> < 0.01), 4 and 8 (<i>P</i> < 0.01), and the number of spontaneous pregnancies CG: 5, and SG: 9 (<i>P</i> < 0.01).	Asthenosperm: after th, the number of cases evaluated as with moderate or marked improvement in the percentage of progressively motile sperm was 3 and 2 (<i>P</i> > 0.05) and 1 and 1 (<i>P</i> > 0.05)	
Wang et al. [35]	2010	A/basic sperm parameters	135 Group A (<i>n</i> = 68) and B (<i>n</i> = 67)	Group A: L-carnitine 2 g/day + vitamin E Group B: vitamin E/3 months	Group A: ↑ % of forward motile sperm (28.6% ± 9.2% to 45.4% ± 11.1%, <i>P</i> < 0.01), the rate of spontaneous pregnancy ↑ (31.1%) than in group B (3.8%) after the treatment (<i>P</i> < 0.01).	Group A: sperm density and the % of the sperm of normal morphology (<i>P</i> > 0.05).	
Plomboni et al. [36]	2008	AT + leukocytosis/sperm parameters, DNA damage (acridine orange)	51 (SG: 36 + CG: 15)	SG: beta-glucan 20 mg, fermented papaya 50 mg, lactoferrin 97 mg, vit. C 30 mg, and vit. E 5 mg, twice per day/3 months	SG: % of morphologically normal sperm (17.0 ± 5.2 to 29.8 ± 6.5) and total progressive motility (19.0 ± 7.8 to 34.8 ± 6.8), ↓ in leukocyte conc. (2.2 ± 0.9 to 0.9 ± 0.2), all <i>P</i> < 0.01	Structural sperm characteristics as well as chromatin integrity were also improved after treatment.	
Balercia et al. [37]	2004	iA (WHO 1999)/basic sperm parameters, seminal plasma and sperm CoQ10, and phosphatidylcholine (PC)	22	CoQ10 200 mg 2x/day/6 months	CoQ10 sem. plasma (ng/mL): 42.0 ± 5.1 to 127.1 ± 1.9 (<i>P</i> < 0.005)) CoQ10 sperm cells (ng/10 ⁶ cells): 3.1 ± 0.4 to 6.5 ± 0.3 (<i>P</i> < 0.05) PC sem. plasma (μM): 1.49 ± 0.50 to 5.84 ± 1.15 (<i>P</i> < 0.05) PC sperm cells (nmol/10 ⁶ cells): 6.83 ± 0.98 to 9.67 ± 1.23 (<i>P</i> < 0.05) Sperm cell motility 9.13 ± 2.50% to 16.34 ± 3.43% after th. (<i>P</i> < 0.05)	Sperm conc. and sperm morphology	

TABLE 1: Continued.

Study/author	Year	Patients/test	Number of patients	Antioxidant/duration of th.	Significant improvement	Nonsignificant improvement	Negative effect
Moslemi and Tavanbakhsh [38]	2011	iAT/semen parameters and pregnancy rates	690 (analysis on 525)	Se 200 µg + vitamin E 400 units/min. 100 days	52.6% (362 cases) total improvement in sperm motility, morphology, or both and 10.8% (75 cases) spontaneous pregnancy versus no treatment (95% confidence interval): 3.08 to 5.52; $P \leq 0.001$	No response to treatment occurred in 253 cases (36.6%)	
Keskes-Ammar et al. [39]	2003	Infertile men/basic sperm parameters, MDA, and serum vitamin E level.	54: SG: 28 (20 analyzed) CG: 26	SG: vitamin E 400 mg + Se 225 µg/day CG: vitamin B 4.5 g/day/3 months	SG: ↓ in MDA concentrations and an ↑ of sperm motility		
Cavallini et al. [40]	2012	Idiopathic OAT/basic sperm parameters and aneuploidy (FISH)	55 (analysis on 33: 22 responder—group 1 + 11 nonresponder—group 2)	L-carnitine 1 g given twice per day + acetyl-L-carnitine 500 mg given twice per day + one 30 mg cinnocicam tablet every 4 days/3 months	Group 1 versus group 2: improvement in morphology and number of aneuploid spermatozoa ($P < 0.01$); ↑ % of biochemical pregnancy after ICSI (54.4% versus 9.1%, $P < 0.01$), clinical pregnancy after ICSI (50% versus 9.1%, $P < 0.01$), and live births (45.4% versus 9.1%, $P < 0.01$)	Numbers of oocytes fertilized and embryos transferred	
Roseff [41]	2002	Subfertile/basic sperm parameters before and after capacitation and mannose receptor binding	19	Pycnogenol 200 mg daily orally/90 days	The mean sperm morphology following Ham's F-10 capacitation ↑ by 38% following th. ($P < 0.001$) and the mannose receptor binding assay scores improved by 19% ($P < 0.005$)	Baseline morphology ↑ after th. by 33%	The mean % change from baseline sperm count after th ↓ nonsignificantly by 10%
Song et al. [42]	2012	Idiopathic OA/basic sperm parameters, DFI (SCSA)	SG: 24 CG: 26	SG: vit. E + xuanju caps CG: vit. E/3 months	SG versus CG: ↓ DFI after th.: 29.57 ± 12.19 versus 34.09 ± 10.32 , $P < 0.05$		
Ménézo et al. [43]	2007	At least TWO previous failures IVF or ICSI, DFI >15%/DFI and the degree of sperm decondensation (SCSA)	58	Vitamins C and E 400 mg each, β-carotene 18 mg, Zn 500 µmol, and Se 1 µmol/90 days	↓DNA fragmentation: -9.1%, $P < 0.0004$		↑ in sperm decondensation (+22.8%, $P < 0.0009$).
Greco et al. [44]	2005	TUNEL >15%/basic sperm parameters, TUNEL	38 (26 OAT + 6 OT + 6 normal)	Vit. C 1g + vit. E 1g/2 months	TUNEL positive sperm: 24.0 ± 7.9 to 8.2 ± 4.3 ($P < 0.001$) Clinical pregnancy after ICSI: from 6.9% to 48.2% ($P < 0.05$) Implantation rate after ICSI: from 2.2% to 19.6% ($P < 0.01$)	Sperm conc: 17.9 ± 16.3 to 18.3 ± 17.9 Sperm motil.: 40.6 ± 24.8 to 39.9 ± 19.0 Normal sperm morph.: 10.5 ± 8.3 to 9.6 ± 0.4 , all $P > 0.05$	

TABLE 1: Continued.

Study/author	Year	Patients/test	Number of patients	Antioxidant/duration of th.	Significant improvement	Nonsignificant improvement	Negative effect
Greco et al. [45]	2005	TUNEL >15%/basic sperm parameters, TUNEL	SG: 32 PG: 32	Vit. C 1g + vit. E 1g/2 months	SG: ↓ fragm. DNA: 22.1 ± 7.7 → 9.1 ± 7.2 ($P < 0.001$)	PG: TUNEL: 22.4 ± 7.8 → 22.9 ± 7.9	
Raigani et al. [46]	2013	OAT/sperm quality, sperm mitochondrial function, sperm chromatin status, semen and blood folate, zinc, B12, TAC, and MDA concentr.	83	Folic acid 5 mg/day ± Zn sulphate 220 mg/day versus placebo/16 weeks	Sperm chromatin integrity (%) ↑ in group receiving only Zn sulphate treatment ($P = 0.048$)	Sperm conc. ↑ in group receiving the combined th. of folic acid and Zn sulphate and also in the group receiving only folic acid th.; ($P = 0.056$ and $P = 0.05$, respectively).	
Tremellen et al. [47]	2007	Male factor infertility, TUNEL >25%/embryo quality, pregnancy and fertilization rate after IVF-ICSI	SG: 36 PG: 16	Menevit (likopen, vit. C, vit. E, Zn, Se, folate, and garlic)/3 months	Pregnancy rate after ICSI in SG: 38.5%, versus PG: 16% ($P = 0.046$)		
Safarinejad et al. [48]	2011	iOAT/semen parameters and TAC of seminal plasma	260 (SG: 130, PG: 130)	Saffron 60 mg/day/26 weeks		No statistically significant improvements in either group in any of the studied semen parameters	
Nadjarzadeh et al. [49]	2011	iOAT/basic sperm parameters, TAC	47	CoQ10 200 mg/day/12 weeks versus placebo	SG: ↑ TAC ($P < 0.05$)	Semen parameters of CoQ10 group	
Comhaire et al. [50]	2000	Infertile men/sperm characteristics, ROS, fatty acids of sperm membrane phospholipids, sperm oxidized DNA (8-OH-dG), and induced AR	27	N-acetyl-cysteine or vitamins A + E and essential fatty acids	↓ ROS, ↑ AR	No improvement in sperm motility and morphology or ↓ of round cells and white blood cells in semen. Sperm concentration ↑ in oligozoosp. men (7.4 ± 1.3 to 12.5 ± 1.9 million/mL).	

Legend: Addyzoa: Gokshura (*Tribulus terrestris*) 200 mg, Ashvagandha (*Withania Sonnifera*) 150 mg, Ashwagandha (*Withania Sonnifera*) 150 mg, Amalaki (*Embellica officinalis*) 75 mg, Balamool (*Sida cordifolia*) 75 mg, Vridhadharu (*Argyrea speciosa*) 75 mg, Shatavari (*Asparagus racemosus*) 75 mg, Shwet musli (*Chlorophytum arundinaceum*) 150 mg, Shuddha kapikachhu (Purified *Mucuna pruriens*) 150 mg, Varahikand (*Tacca aspera*) 30 mg, Chopchin (*Smilax china*) 30 mg, Vidarikand (*Ipomoea digitata*) 30 mg, Munjatak (*Eulophia campestris*) 15 mg, Purnachandrodaya rasa 45 mg, Suvarnavang 30 mg, Muktashtukti bhasma 30 mg, Suvarnamakshik bhasma 30 mg, Shilajit shuddha 30 mg, Abhrak bhasma 15 mg, Makardhwaj rasa 15 mg, Rasa sindur 5 mg, AR: acrosome reaction; CG: control group; DDS: DNA degraded sperm; DFI: DNA fragmentation index; Fertilovit Mplus: L-citrulline (20.2 %), L-carnitine-L-tartrate, D-alpha-tocopheryl acetate, hydroxypropyl methylcellulose (capsule coating), acidifier tartaric acid, L-ascorbic acid (6.7%), parting compound silicon dioxide, calcium carbonate, lycopene, N-acetyl-L-cysteine, glutathione (reduced), corn starch, zinc oxide, coenzyme Q10, vegetable oil, shellac coating, pteroyl-L-glutamate, sodium selenite, coloring agent titanium dioxide (capsule), coloring agent orange yellow S (capsule); CoQ10: coenzyme Q10, FISH: fluorescent in situ hybridization; FSH: follicle-stimulating hormone; ICSI: intracytoplasmic sperm injection; iOAT: idiopathic OAT; IVF: in vitro fertilization; LH: luteinizing hormone; MDA: malondialdehyde; MSOME: motile sperm organelle morphology examination; OAT: oligoasthenoteratozoospermia; PG: placebo group; ROS: reactive oxygen species; Sairei-to: a Chinese herbal drug; SCSA: sperm chromatin structure assay; Se: selenium; SG: study group; T: testosterone; TAC: total antioxidant capacity; TUNEL: TdT (terminal deoxynucleotidyl transferase)—mediated dUTP nick-end labeling; Xuanju: *Formica fusca*, Herba epimedii, and *Fructus lycii*; Zn: zinc.

vitamin E with degree of DFI of 34.09 ± 10.32 ($P < 0.05$) [42]. Greco et al. [44, 45] had proved that 1 g of vitamin C and 1 g of vitamin E together taken for 2 months significantly decreased the degree of DNA fragmentation from 22.1 ± 7.7 to 9.1 ± 7.2 ($P < 0.001$) [45]. Raigani et al. showed that zinc sulphate significantly improved sperm chromatin integrity [46].

3.5. Pregnancy Rate. CoQ10 [24], clomiphene citrate with vitamin E [26], lycopene [31], L-carnitine with vitamin E [34, 35], and selenium with vitamin E [38] significantly improved spontaneous pregnancy rates during duration of treatment, while L-carnitine with cinnoxycam [40] and vitamins C and E together [44] significantly improved pregnancy rates per cycle after assisted reproductive technology with intracytoplasmic sperm injection (ICSI). Ghanem et al. proved higher spontaneous pregnancy rate in 30 participants after the intake of combination of 25 mg clomiphene citrate and 400 mg vitamin E per day for 6 months (36.7%) than in placebo group (13.3%) with $P = 0.037$ [26]. L-Carnitine, 2 g, with vitamin E taken for 3 months improved spontaneous pregnancy rate to 31.1% compared to vitamin E group with pregnancy rate of 3.8% ($P < 0.01$) [35]. Another example in study by Greco et al. confirmed higher pregnancy rate after 2 months therapy with 1 g of vitamin C and 1 g of vitamin E daily. After ICSI clinical pregnancy rate was 48.2% after therapy versus 6.9% before therapy ($P < 0.05$) [44].

3.6. Negative or No Effect on Sperm Parameters. In this review we find out also rare negative effects of antioxidants on sperm parameters or no effect. Pycnogenol caused nonsignificant fall in baseline sperm count by 10% [41]. Similarly, treatment with vitamins C and E, β -carotene, zinc, and selenium significantly increased sperm decondensation [43]. Large research on saffron showed no statistically significant improvements in any of the studied semen parameters [48].

3.7. Other Parameters. We looked at the basic sperm parameters but there were also many other positive influences; for example, CoQ10 and pentoxifylline caused improvements in total antioxidant capacity and acrosome reaction [22, 25, 49, 50]; FSH value [22, 23] decreased after CoQ10 treatment, semen leucocyte concentration decreased [36], and level of ROS [50] decreased after antioxidant mixtures. Antioxidants protect unsaturated fatty acids and so provide fluidity that is necessary for membrane fusion events like the acrosome reaction. Although hormonal abnormalities are not always evident, iOAT is sometimes associated with lower serum testosterone and inhibin levels and higher serum estradiol, LH, and FSH levels [55, 56]. The increased serum FSH level in men with azoospermia or severe oligozoospermia indicates damaged seminiferous tubule [57] and is inversely associated with sperm concentration, motility, and morphology [58]. ROS has been found in the seminiferous tubules and seminal plasma of most patients with iOAT [59]. Decreased levels of ROS due to antioxidant consumption can cause fall in serum FSH level. Leukocytes are potential source of ROS and due to protective influence of antioxidants their concentration may decrease [15]. In addition, studies have found an increase in

inhibin B value [23] and in superoxide-dismutase- (SOD-) like and catalase activity [21, 25], which among others represent the total antioxidant capacity of seminal plasma [60]. Inhibin B is positively correlated with sperm concentration and is, like FSH, thought to be a marker of spermatogenesis and Sertoli cell function [61, 62].

4. Conclusions

Most of the published studies were randomized and placebo controlled. The majority of studies confirmed beneficial effect of different antioxidants on at least one of the semen parameters and the biggest effect was determined on sperm motility. In many of these trials combinations of more antioxidants were assessed. The optimal dosages of one or more antioxidants were not defined.

Most commonly antioxidants studied were vitamin E, vitamin C, selenium, CoQ10, N-acetyl-cysteine, L-carnitine, and zinc and their favorable effect was confirmed. According to this review favorable effects on iOAT have been determined with CoQ10, vitamin E, selenium, and also vitamin C and N-acetyl-cysteine treatments. In case of oligozoospermia vitamin E and CoQ10 were most often proved to be effective. Favorable effects on asthenozoospermia have most often been determined with vitamin E, CoQ10, and selenium treatments. In teratozoospermia selenium and CoQ10 treatments were most often proved to be effective. In addition, combination of vitamin C and E showed the biggest favorable effect on DNA fragmentation; similar effects were determined with zinc and selenium treatments.

In conclusion, antioxidants play an important role in protecting semen from ROS and can improve basic sperm parameters in case of idiopathic oligoasthenoteratozoospermia.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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Research Article

Time-Lapse Dynamics of the Mouse Oocyte Chromatin Organisation during Meiotic Resumption

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In the mammalian oocyte, distinct patterns of centromeres and pericentromeric heterochromatin localisation correlate with the gamete's developmental competence. Mouse antral oocytes display two main types of chromatin organisation: SN oocytes, with a ring of Hoechst-positive chromatin surrounding the nucleolus, and NSN oocytes lacking this ring. When matured to MII and fertilised, only SN oocytes develop beyond the 2-cell, and reach full term. To give detailed information on the dynamics of the SN or NSN chromatin during meiosis resumption, we performed a 9 hr time-lapse observation. The main significant differences recorded are: (1) reduction of the nuclear area only in SN oocytes; (2) ~17 min delay of GVBD in NSN oocytes; (3) chromatin condensation, after GVBD, in SN oocytes; (4) formation of 4-5 CHCs in SN oocytes; (5) increase of the perivitelline space, ~57 min later in NSN oocytes; (6) formation of a rosette-like disposition of CHCs, ~84 min later in SN oocytes; (7) appearance of the MI plate ~40 min later in NSN oocytes. Overall, we described a pathway of transition from the GV to the MII stage that is punctuated of discrete recordable events showing their specificity and occurring with different time kinetics in the two types of oocytes.

1. Introduction

In the nucleus of eukaryotic cells, chromosomes occupy distinct territories whose position may change during the cell cycle or cell differentiation [1–4]. Entire chromosomes, sub-chromosomal regions, and genes change their nuclear localisation during differentiation to acquire a cell-type-specific spatial organisation, contributing, as part of the epigenome, to the regulation of the cell functions [5–8]. Centromeres and pericentromeric constitutive heterochromatin (CHC) of mammalian cells tend to gather to form chromocenters. The degree of centromere clustering varies depending on the cell type, cell-cycle phase, or stage of differentiation [9–14]. Lineage-specific centromere associations into chromocenters have been reported during somatic [9, 15, 16], male [17],

and female [18, 19] germ cell differentiation. Within the nucleus, the nucleolus is a major attractive compartment for heterochromatic regions such as inactive X-chromosome, regions enriched in repressed genes, and pericentromeric repeated sequences [20]. Altogether, these studies indicate that a specific nuclear localisation of these chromatin traits is required for a correct genome functioning at different cell-cycle phases and different stages of cell differentiation [21].

To this regard, the mammalian oocyte is a particularly intriguing cell model study as distinct patterns of centromeres and pericentromeric CHC localisation correlate with the gamete meiotic and developmental competence. When stained with Hoechst 33342 (Ho), a supravital fluoro-chrome that preferentially binds to the AT-rich regions of the genome, fully grown germinal vesicle (GV) mouse

antral oocytes display two main different types of chromatin organisation: Surrounded Nucleolus (SN) oocytes, with a ring of Ho-positive chromatin surrounding the nucleolus and Nonsurrounded Nucleolus (NSN) oocytes, lacking the ring and with a more dispersed chromatin [22–24]. A bold of experimental evidence has shown that, following their isolation from the ovarian surface, SN and NSN antral oocytes display a different meiotic competence, with 82% or 45% of SN and NSN oocytes, respectively, reach the metaphase II (MII) stage [25]. Then, after fertilisation, only SN oocytes may develop to term, whereas NSN oocytes arrest at the 2-cell stage [25–28].

The two different nuclear phenotypes underlie specific transcriptional and translational programmes central to the acquisition of a correct developmental competence or developmental failure [29–32]. Their distinct chromatin organisations, which are found in most of the mammals studied (for a review see [33]), reflect the pattern of localisation and arrangements of the nucleolar organising region- (NOR-) bearing chromosomes and of the nuclear and perinucleolar localisation of centromeres and their associated pericentromeric CHC occurring during folliculogenesis [18, 19].

The crux of the present study is to contribute detailed information on the morphological transformations occurring to the nuclear organisation of the SN or NSN chromatin during meiosis resumption. We conducted a live observation of GV oocytes while progressing *in vitro* towards the MII stage, with the aim of giving an accurate description of the dynamics of this transition. To this end, we isolated fully grown antral oocytes from the ovaries of females primed with PMSG and, after staining with Ho, we performed a comprehensive 9 hr time-lapse imaging of both SN and NSN oocytes. The results describe a pathway of transition from the GV to the MII stage that is punctuated of discrete recordable events showing their specificity and occurring with different time kinetics in the two types of oocytes.

2. Materials and Methods

2.1. Animals. Four- to six-week-old CD1 female mice were used for this study (Charles River, Como, Italy). Mice were maintained by the Department of Animal Facility according to the Guide for Care and Use of Laboratory Animals, under conditions of 21°C temperature and a dark/light cycle of 12/12 hours. This research has been performed after the approval of the Animal Ethics Committee of the University of Pavia and carried out in strict accordance with the protocol approved by the European (n. 86/609/CEE) and Italian (n. 116/92, 8/94) legislation.

2.2. Hormonal Treatment. Forty-eight hours before sacrifice and oocytes isolation, female mice were intraperitoneally injected with 3.75 IU Folligon (PMSG, Intervet Italia, Segrate, Italy).

2.3. Isolation of Fully Grown Antral Oocytes. Fully-grown antral oocytes were collected in M2 medium by puncturing the ovarian surface with a sterile insulin needle. Then,

they were freed from surrounding cumulus cells by gently pipetting in and out with a mouth-controlled hand-pulled glass Pasteur micropipette.

2.4. Classification and Maturation of Antral Oocytes to the MII Stage. Immediately, after their isolation from the ovary, cumulus-free antral oocytes were transferred into 20 μ L droplets of Ho fluorochrome (0.05 μ g/mL; Sigma-Aldrich, Milano, Italy, cat. N. B2261) in M2 medium for 12 min at room temperature. Details on the method of classification of SN or NSN oocytes are given in [24]. Three-four oocytes were transferred in each of four 2 μ L α -MEM drops (Life Technologies, Monza, Italy, cat. N. M4526) placed at the centre of a 3.5 cm glass-bottom Petri dish (WillCo Wells B.V., Amsterdam, The Netherlands, cat. N. GWSt-3522) and covered with mineral oil (Sigma-Aldrich, cat. N. M8410). α -MEM was supplemented with 5% heat-inactivated foetal bovine serum (Life Technologies, cat. N. 10270106), 2 mM L-Glutamine (Life Technologies, cat. N. 25030), 5 mM Taurine (Sigma-Aldrich, cat. N. T0625), and 36 μ g/mL sodium pyruvate (Sigma-Aldrich, cat. N. P4562). Oocytes were *in vitro* matured (IVM) for a total of 15 hr inside a BioStation IM (Nikon, Torino, Italy) at 37°C, under a 5% CO₂ humidified atmosphere. At the end of the culture period, first polar body (PBI) extrusion was assessed by monitoring oocytes under an Olympus SZX9 (Olympus, Milano, Italy) stereomicroscope.

2.5. Time-Lapse Analysis. Time-lapse analysis was performed over a 9 hr total recording. Bright field and fluorescence images of oocytes were taken at 8 min time intervals and for time-lapse segments of a maximum of 1.5–2 hr; this segmentation was decided after preliminary experiments indicating that an exposure to UV irradiation prolonged beyond the 2 hr could affect the oocyte maturation. To cover the whole 9 hr recording period, five time-lapse segments were designed: from 0 to 2 hr (12 SN and 7 NSN oocytes were recorded), from 2 to 3.5 hr (6 SN and 5 NSN oocytes), from 3.5 to 5 hr (10 SN and 12 NSN oocytes), from 5 to 7 hr (9 SN and 6 NSN oocytes), and from 7 to 9 hr (20 SN and 7 NSN oocytes), for a total of 57 SN and 37 NSN oocytes analysed. At each 8 min time interval, a 15 sections Z-stack, 4 μ m spaced, was arranged. In order to produce the movies, all the time-lapse images acquired with the BioStation during each time segment analysed were subsequently imported into the Image J software and then converted into a file .avi (7 frame per second). The GV (nucleus) area was measured using the CellSens Dimension software (Olympus). Since the GV shape is never that of a circle, this software allows drawing a Region Of Interest (ROI) by connecting a number of points that are chosen by the operator and then it calculates its area (pixel²).

2.6. Statistics. Differences in the timing in which meiotic events occurred were evaluated with Student's *t*-test and Mann-Whitney *U*. Changes in the nuclear area were evaluated using Student's *t*-test. Data analysed with the SigmaStat 3.5 software were considered significantly different when *P* < 0.05.

TABLE 1: Timing of events occurring during meiosis resumption of antral oocytes cultured *in vitro*.

Type of event	Mean \pm S.D. in minutes SN oocytes	NSN oocytes	P value*
GVBD	34.2 \pm 8.1	51.4 \pm 19.0	<0.05
Increase of the perivitelline space	24.0 \pm 10.6	96.0 \pm 24.0	<0.001
Formation of 4-5 CHCs	92.4 \pm 9.9	ND**	—
Formation of 8–10 small pericentromeric regions (rosette)	223.2 \pm 20.8	139.2 \pm 20.9	<0.01
MI plate	350.2 \pm 34.6	390.7 \pm 20.5	<0.05
Anaphase I	483.6 \pm 33.0***	ND	—
Extrusion of polar body I and formation of MII plate	520.7 \pm 20.7***	ND	—

* Comparison of SN versus NSN.

** Not detected.

*** Calculated on 55% of oocytes that reached anaphase I within the 9 hr recording period.

3. Results

In this study we observed the morphological changes that occur to NSN and SN fully grown antral GV oocytes (bright field) and to the organisation of their Ho-positive chromatin (fluorescence) during the transition towards the MII stage. The details that are given hereafter are representative of the observations made for each of the time-lapse phases recorded of those oocytes that reached MII.

3.1. Time-Lapse Imaging from 0 to 120 Minutes. During the first 2 hr of SN oocytes culture, bright-field observations showed that the germinal vesicle break down (GVBD) occurs on average 34.2 ± 8.1 min since the starting of the recording (Table 1). GVBD is preceded by a 12.4% reduction ($P < 0.01$) of the GV area (Figure 1(a) (a–d)) and culminates with the disassembly of the nuclear envelope and dismantle of the characteristic rounded shape (Figure 1(a) (e); see Additional file 1 available online at <http://dx.doi.org/10.1155/2014/207357>). Around the nucleus we observed a thin and dark rim which assumes a granular pattern, that is, small black dots that first gather along the perimeter and then, with the disassembly of the nuclear envelope, move towards the nucleus area centre that remains visible, throughout the 2 hr observation, as a hollow (nuclear hollow, NH) (Figure 1(a) (e–n) and enlargements).

Almost coincidentally with the formation of the NH (24.0 ± 10.6 min), we observed a sudden detachment of the zona pellucida (ZP) at one side of the oocyte (Table 1; Figure 1(a) (d), (e), arrow; Additional file 1), likely due to a slight contraction. As a consequence, the size of the perivitelline space increases and is maintained as such throughout the remaining culture period. Whilst the increase of the perivitelline space was a feature of the gametes at this stage of maturation, in three out of twelve oocytes analysed we did not record this characteristic.

When observed under ultraviolet (UV) light, the typical SN chromatin configuration (Figure 1(a) (a') and enlargement) is maintained unaltered during the first 29.1 ± 14.0 min (Figure 1(a) (a'–d')); then, abruptly during the following 8 min and concomitantly with the GVBD (Figure 1(a) (e') and enlargement), we recorded an increased chromatin

condensation in the area where the nucleolus was positioned (Additional file 2). During the remaining culture period, with the disappearance of the ring of Ho-positive chromatin, 4-5 large CHCs become visible (92.4 ± 9.9 min) (Table 1) (Figure 1(a) (l'–p')), with the exception of few thread-like chromatin structures visible among the CHCs (Figure 1(a) (l'), arrowhead in enlargement).

In NSN oocytes (Figure 1(b) (a') and enlargement), the features described above displayed overall more variable patterns. The GVBD occurred at 51.4 ± 19.0 min since the beginning of the culture period (Table 1; Figure 1(b) (j)), significantly later ($P < 0.05$) than that of SN oocytes, although in one oocyte it occurred at 16 min and in another occurred as late as 72 min. The rim of small black dots coalesced towards the centre of the NH at later stages of maturation compared to SN oocytes (Figure 1(b) (l) and enlargement). The size of the perivitelline space increased, but much later (96.0 ± 24.0 min; Table 1) ($P < 0.001$) compared to SN oocytes, and only in three out of seven oocytes analysed.

Under the UV light, throughout the 0 to 120 min interval, we recorded an almost static image showing the presence of 4/5 small Ho-positive CHC around the nucleolus (Figure 1(b) (a'–p'); Additional file 3). A visible change was observed at the time of GVBD when, at the disappearance of the nucleolus, the CHCs slightly moved towards the periphery (Figure 1(b) (i') and enlargement).

3.2. Time-Lapse Imaging from 128 to 216 Minutes. The size and shape of SN oocytes during this time-lapse period remained mainly unchanged and, at the bright field, we did not observe specific marker features.

Under UV light, from the 128 min onwards, we recorded a progressive partial decondensation of the CHCs (Figure 2(a) (b'–m'); see enlargement: arrow, large CHC; arrowhead, small CHC; Additional file 4). In NSN oocytes, following the first 120 min of culture when chromatin organisation does not change, chromosomes appear abruptly (139.2 ± 20.9 min of culture (Table 1; Figure 2(b) (b') and enlargement), much earlier than in SN oocytes (see below) and assume a “rosette-like” organisation (Figure 2(b) (e') and enlargement) that is maintained up to the end of this recording time segment (Additional file 5).

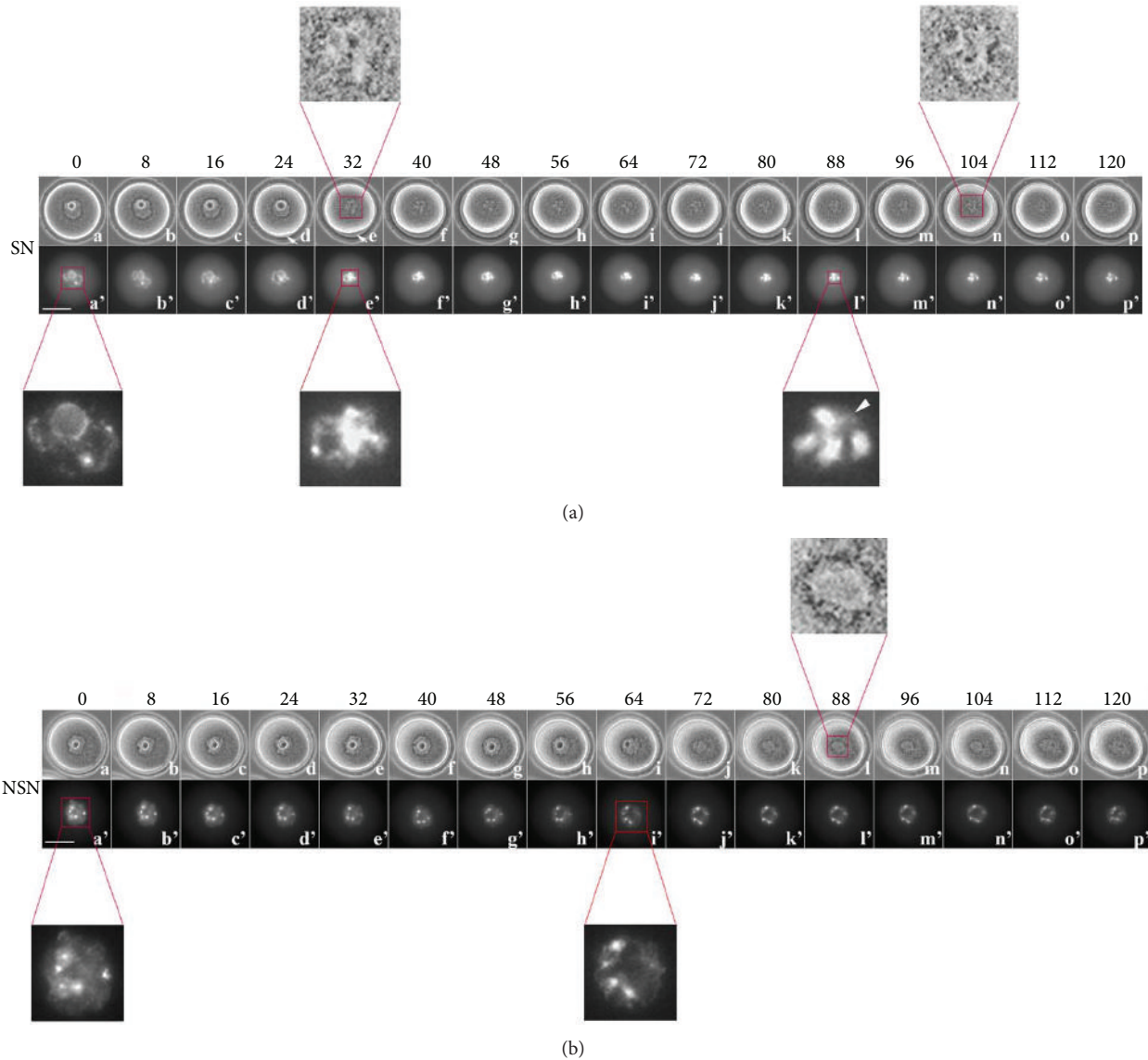


FIGURE 1: Time-lapse imaging during the first 120 minutes of oocyte culture. (a) SN oocyte, (a–p) bright field; (a'–p') UV fluorescence describing the changes to the Ho-positive chromatin within the nucleus. Arrow in (e) and (d) shows increase of perivitelline space. Arrowhead in (l') enlargement thread-like chromatin structures is visible among the pericentromeric CHCs. (b) NSN oocyte, (a–p) bright field; (a'–p') UV fluorescence. Bar: 40 μ m.

3.3. Time-Lapse Imaging from 224 to 296 Minutes. In SN oocytes, 8–10 Ho-positive regions arrange to form a “rosette-like” distribution after 223.2 ± 20.8 min culture (Table 1; Figure 3(a) (b'–k') and enlargement), later ($P < 0.01$) than NSN oocytes. This organisation is maintained throughout this recording time segment (Additional file 6).

Chromosomes of NSN oocytes maintain the “rosette-like” disposition acquired during almost the whole period (Figure 3(b) (b'–k')), although, by the end, they begin moving towards the oocyte surface (Additional file 7).

3.4. Time-Lapse Imaging from 304 to 416 Minutes. This time-lapse segment is characterised by the formation of a clear MI plate, which appears earlier ($P < 0.05$) in SN ($350.2 \pm$

34.6 min, Figure 4(a) (d') and enlargement) than NSN (390.7 ± 20.5 min, Figure 4(b) (i') and enlargement) oocytes. The MI plate changes localisation in both SN (Additional file 8) and NSN (Additional file 9) oocytes (Figure 4(a) (d'–p') and Figure 4(b) (i'–p')).

3.5. Time-Lapse Imaging from 424 to 536 Minutes. Anaphase I (Table 1; Figure 5(a) (b'), Figure 5(b) (h') and enlargements), with two separated chromosome sets and PBI extrusion (SN: Table 1; Figure 5(a) (h)) (Additional files 10 and 11), is the main feature of this time-lapse segment. Although all the SN or NSN oocytes analysed reached the MII phase, the latter was attained before the end of the 9 hr recording period (480 min) by 55.0% SN or 14.3% NSN oocytes, respectively.

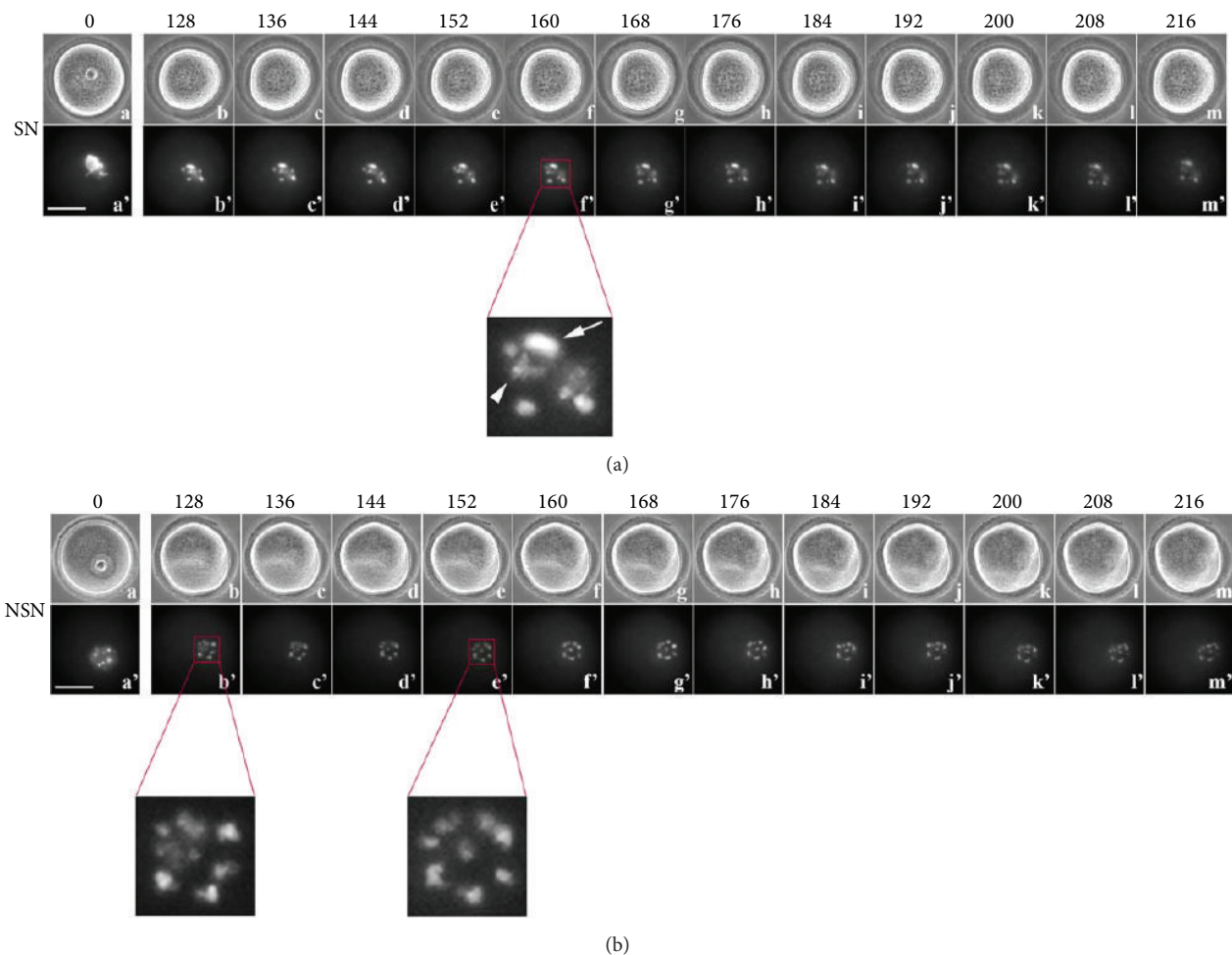


FIGURE 2: Time-lapse imaging from 128 to 216 minutes of oocyte culture. (a) SN oocyte, (a–m) bright field; (a'–m') UV fluorescence. Arrow in (f') enlargement, large CHC; arrowhead, small CHC. (b) NSN oocyte, (a–m) bright field; (a'–m') UV fluorescence. Bar: 40 μ m.

The remaining 45.0% or 85.7% SN or NSN, respectively, attained the MII after the 9 hr of time-lapse recording, within the 15 hr of IVM.

4. Discussion

In its reductionist layout, the experimental application of the SN/NSN model is a powerful tool that allows having, at one's disposal, ovarian oocytes of known developmental competence or incompetence. Staining of the chromatin with the fluorochrome Ho gives the opportunity to identify within a pool of oocytes isolated from the antral compartment of the ovary those that may develop to term (SN) from those that certainly arrest development soon after fertilisation (NSN) [25–28]. This early classification allowed us to focus on emerging differences between developmentally competent and incompetent oocytes, while they are maturing *in vitro* from the GV to the MII stage. A first difference that emerges is the significant longer time, almost doubled, that the NSN oocytes spend at the diplotene stage before undergoing GVBD (NSN: ~51 min versus SN: ~34 min). When GVBD is almost completed and the nuclear envelope is dismantled,

we observe a rim of black dots, seen under bright field in both types of oocytes, that represents mitochondria clearly visible for their autofluorescence when analysed at 440–490 nm wavelength ([34, 35]; our unpublished observations). Then, by the time the oocyte reaches the MII phase, these mitochondria disperse within the ooplasm and become invisible when observed at the bright field ([34, 35]; our unpublished observations). These early events in meiosis resumption are accompanied by significant rearrangements of the cytoskeleton [36] that we could clearly record in SN, but less extensively in NSN oocytes, as a pulse contraction of the gamete itself. This shrinkage brings, as a consequence, to an enlargement of the perivitelline space on the one side of the oocyte, increase that is maintained as such throughout the remaining culture period.

The typical chromatin organisation of SN and NSN oocytes is maintained unaltered and distinct during the whole diplotene stage. Then, coincidentally with the GVBD and the beginning of diakinesis, the SN chromatin undergoes numerous changes, instead the NSN chromatin preserves its original organisation for much further (up ~140 min). The NSN nucleus maintains a steady chromatin organisation

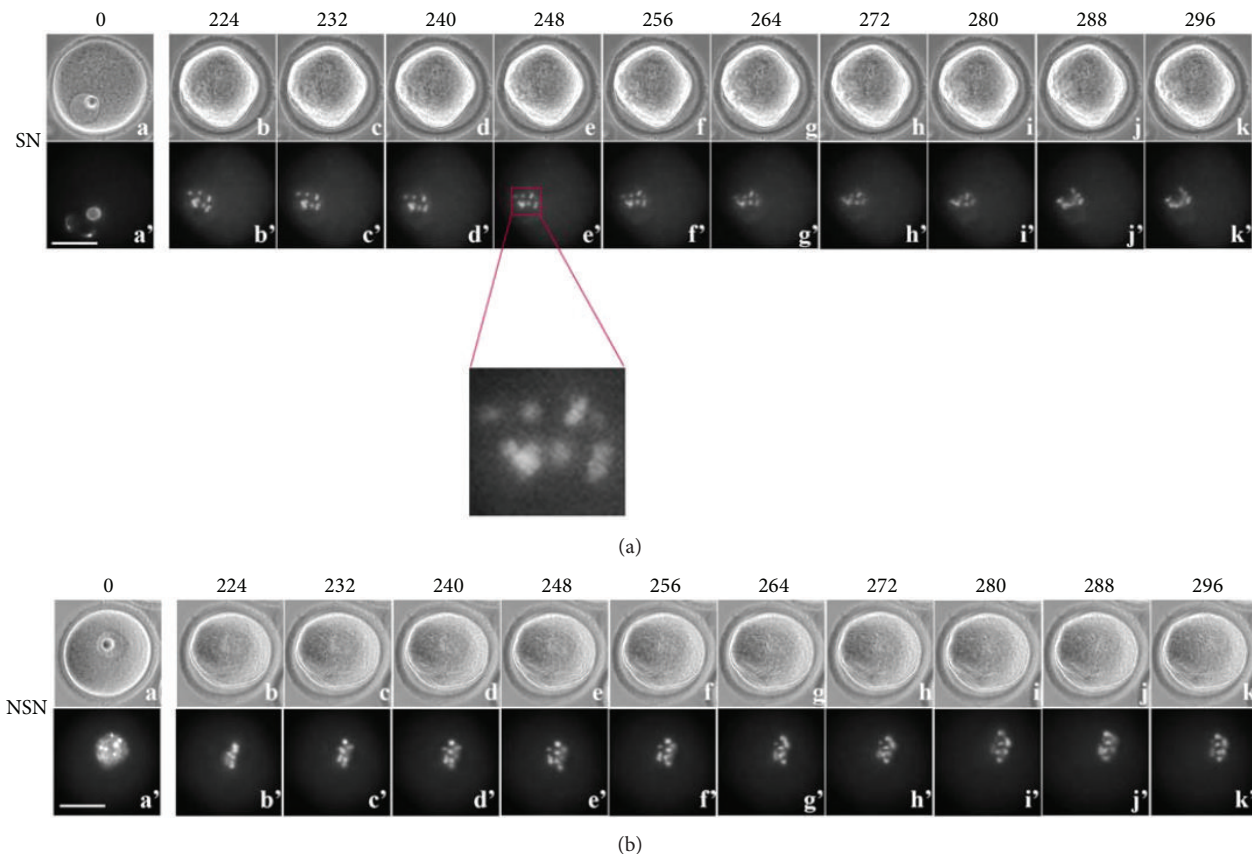


FIGURE 3: Time-lapse imaging from 224 to 296 minutes of oocyte culture. (a) SN oocyte, (a–k) bright field; (a'–k') UV fluorescence. (b) NSN oocyte, (a–k) bright field; (a'–k') UV fluorescence. Bar: 40 μ m.

with 4/5 small Ho-positive CHCs around the nucleolus, corresponding to the pericentromeric area of NOR-bearing chromosomes [18]. Instead, the SN chromatin abruptly condenses (~ 30 min) around the nucleolar area; then, 4/5 CHCs emerge and become larger in size and more separated one from the other, marking the end of diakinesis and the beginning of the following prometaphase (~ 100 – 120 min) [37]. The formation of the CHCs in SN oocytes has been explained with the gathering around the nucleolus of the pericentromeric regions of the 40 telocentric chromosomes of the mouse karyotype [18]. Then, these large CHCs become smaller in size and increase in number, likely as a consequence of the drifting away of chromosomes that later will begin to appear clearly visible as single entities arranged in a “rosette-like” organisation, a disposition of the chromosomes that marks the passage towards the MI phase.

Although these results show that the rosette-like figure is detected ~ 80 min earlier in NSN compared to SN oocytes, the transition to MI occurs earlier in SN (~ 350 min) compared to NSN (~ 390 min) oocytes, suggesting a longer permanence in prometaphase for the latter gametes. The extended prometaphase in NSN oocytes may be explained with chromosome lagging in the congression towards the MI plate formation and may be causal to the about 4-fold higher aneuploidy rate that we described in NSN compared to SN

oocytes in females that have undergone the same hormonal treatment [38]. The correlation between chromosome lagging and aneuploidy will be further analysed with a more detailed time-lapse analysis of the first meiotic division.

In summary, NSN oocytes undergo chromatin changes, distinct from those of SN oocytes, which prepare the genome to accomplish the following meiotic phases and reach MII. We observed a longer GV-to-MII transition in NSN oocytes that reach the M-phase without the characteristic gathering of heterochromatin regions around the nucleolus [23]. Although we cannot identify a specific cause for the observed delay, a number of features that have been described may build up to a comprehensive picture of the biological nature of these two different antral oocytes. Interestingly, they display a different epigenetics status; that is, the SN chromatin configuration has higher levels of CpG methylation, histone acetylation (H4K5ac and H4K12ac), and methylation (H3K9me2) [39] which may be crucial to the dynamics of the large scale chromatin remodelling occurring soon after meiosis resumption. Interestingly, delayed transition was also described in SN oocytes treated with the histone deacetylase inhibitor trichostatin A [40], which prevents the onset of the global deacetylation occurring soon after meiosis resumption, indicating that perhaps NSN oocytes may present a lower or even damaged deacetylation activity. In addition to these described

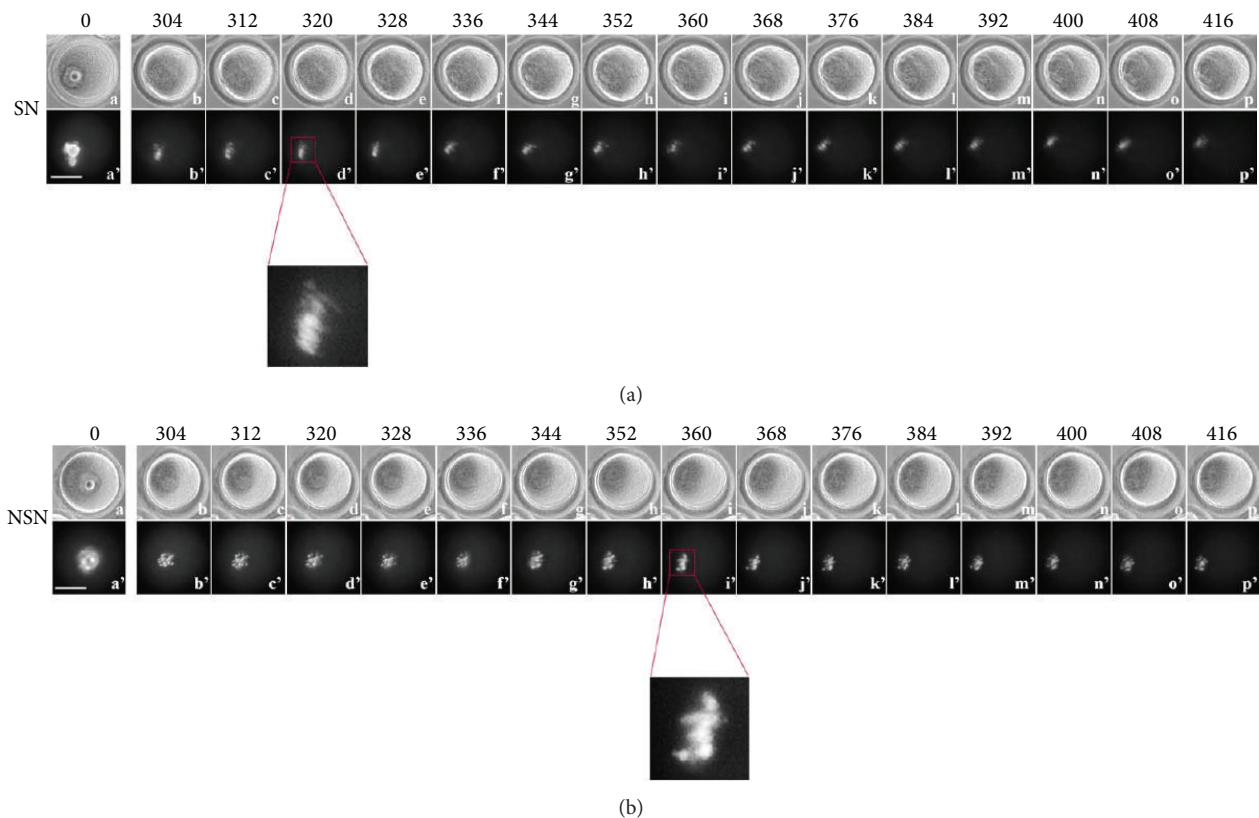


FIGURE 4: Time-lapse imaging from 304 to 416 minutes of oocyte culture. (a) SN oocyte, (a–p) bright field; (a'–p') UV fluorescence. (b) NSN oocyte, (a–p) bright field; (a'–p') UV fluorescence. Bar: 40 μ m.

differences, our own whole transcriptome microarrays studies show that NSN oocytes exhibit upregulation of transcriptional networks associated with mitochondrial dysfunction and apoptosis and downregulation of cell cycle transcripts [29].

When considered together with a number of recent molecular data, our morphological observations help to further understand the biological significance of these two types of oocytes within the ovary. Morphological and molecular data speak in favour of a separation of the maturation pathways of SN and NSN oocytes and possibly a distinct fate within the ovary. Microarrays studies demonstrated the presence in SN oocytes of a transcriptional network (TN) regulated by the oocyte-specific transcription factor OCT4 (OCT4-TN), whose downregulation in NSN oocytes plays a key function in a sequence of molecular events that lead to their developmental arrest [29]. From these studies, OCT4 emerges as a crucial regulator of the events that govern the establishment of the developmental competence of mouse oocytes [29, 31]. Mostly important is the pattern of expression of OCT4, which remains confined to oocytes with an SN type of chromatin configuration from the beginning to the end of oocyte growth, whereas it is downregulated in NSN oocytes throughout folliculogenesis [30]. A similar profile of expression is shown by other OCT4-regulated genes, including STELLA (DPPA3) [30], another oocyte-specific

transcription factor whose lack of expression leads to a developmental arrest mainly at the 2-cell stage [30, 41].

5. Conclusions

In this study, we have minutely described and filmed, using time-lapse imaging, modifications to the oocyte morphology and to its chromatin organisation. The overall picture that comes to light is that of a pathway of transition from GV to MII for the two types of oocytes which is punctuated of discrete recordable events that show their specificity and occur with different time kinetics. The main significant differences recorded during oocyte maturation are (1) a reduction of the nuclear area that occurs before the GVBD, significant only for SN oocytes; (2) a ~17 min delay of the GVBD in NSN oocytes; (3) an increased chromatin condensation, soon after the GVBD, that occurs only in SN oocytes; (4) the formation of 4-5 CHCs only in SN oocytes; (5) an increase of the perivitelline space that occurs ~57 min later in NSN oocytes; (6) the formation of a rosette-like disposition of the pericentromeric regions that takes place ~84 min later in SN oocytes; (7) the MI plate appears ~40 min later in NSN oocytes.

Altogether, morphological and molecular data of earlier studies build up to a model of mammalian ovary that

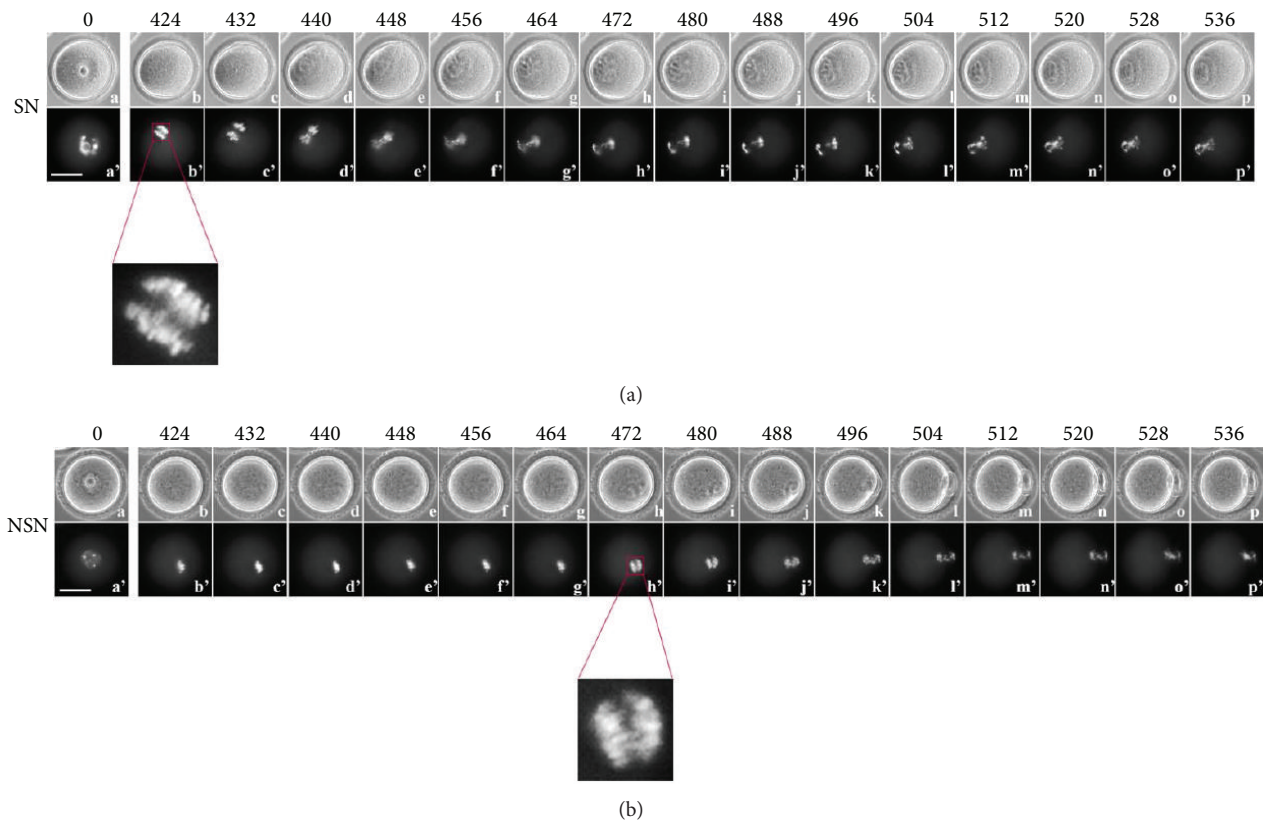


FIGURE 5: Time-lapse imaging from 424 to 536 minutes of oocyte culture. (a) SN oocyte, (a–p) bright field; (a'–p') UV fluorescence. (b) NSN oocyte, (a–p) bright field; (a'–p') UV fluorescence. Bar: 40 μm .

envisages the coexistence of follicles enclosing oocytes that are potentially developmentally competent or incompetent and both capable of growth and meiotic differentiation, at least *in vitro*. This model raises numerous questions, including whether growing follicles containing developmentally incompetent NSN oocytes may be rescued, that is, guided to acquire the SN chromatin organisation and whether this is paralleled by the acquisition of a developmental competent state. The answer to this question would improve our understanding of the yet poorly known biology of the mammalian ovary and would carry positive clinical implications to enhanced assisted reproductive technologies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

BACs-on-Beads Technology: A Reliable Test for Rapid Detection of Aneuploidies and Microdeletions in Prenatal Diagnosis

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The risk of fetal aneuploidies is usually estimated based on high resolution ultrasound combined with biochemical determination of criterion in maternal blood, with invasive procedures offered to the population at risk. The purpose of this study was to investigate the effectiveness of a new rapid aneuploidy screening test on amniotic fluid (AF) or chorionic villus (CV) samples based on BACs-on-Beads (BoBs) technology and to compare the results with classical karyotyping by Giemsa banding (G-banding) of cultured cells in metaphase as the gold standard technique. The prenatal-BoBs kit was used to study aneuploidies involving chromosomes 13, 18, 21, X, and Y as well as nine microdeletion syndromes in 321 AF and 43 CV samples. G-banding of metaphase cultured cells was performed concomitantly for all prenatal samples. A microarray-based comparative genomic hybridization (aCGH) was also carried out in a subset of samples. Prenatal-BoBs results were widely confirmed by classical karyotyping. Only six karyotype findings were not identified by Prenatal-BoBs, all of them due to the known limitations of the technique. In summary, the BACs-on-Beads technology was an accurate, robust, and efficient method for the rapid diagnosis of common aneuploidies and microdeletion syndromes in prenatal samples.

1. Introduction

Birth defects are responsible for many cases of infant mortality and morbidity around the world [1], with about seven percent of all neonatal deaths being caused by congenital anomalies [2]. It is estimated that six percent of these congenital defects are due to aneuploidies and nearly one in 200 newborns is affected [3].

These congenital defects are caused by chromosomal aneuploidies or monogenic disorders; nevertheless, environmental causes such as fetal infections, environmental teratogens, or micronutrient deficiencies could be hidden by the low percentages for these factors. In fact, most of these defects are due to the combined effects of environmental and genetic factors [2].

The risk of fetal aneuploidies is usually estimated based on high resolution ultrasound scans combined with biochemical determinations in maternal blood samples. The most important biochemical markers measured in the first trimester of pregnancy are the free beta-subunit of human chorionic gonadotropin ($f\beta$ -hCG) and pregnancy-associated plasma protein A (PAPP-A), and the most important ultrasound marker is the measurement of nuchal translucency. In the second trimester, alpha-fetoprotein (AFP) and beta-human chorionic gonadotropin (β hCG) are also measured [2, 4]. In fact, second-trimester screening has been discontinued lately because it resulted in the worst detection rates (around 70% estimated detection rates for a 5% false positive rate for Down syndrome). Nevertheless, second-trimester screening would be only used when women are attending to the first medical

TABLE 1: Description of the aneuploidies and microdeletion syndromes included in the Prenatal BoBS kit.

Syndrome	Frequency of occurrence	Lifespan	Mental retardation	Severe medical symptoms
Down syndrome (21)	1/750–800	50 years	Mild to moderate	–/+
Patau syndrome (13)	1/6,000	4 days	Severe	++
Edwards syndrome (18)	1/10,000	2.5 days	Severe	++
Triple X syndrome (XXX)	1/1,000	Normal	No	–
Klinefelter syndrome (XXY)	1/500–1,000	Normal	No	–
XYY syndrome (XYY)	1/1,000	Normal	No	–
Turner syndrome (X0)	1/2,500	Slightly reduced	Mild to moderate	–/+
Wolf-Hirschhorn (4p16, 3)	1/50,000	Limited	Moderate to severe	+
Cri du Chat (5p15, 3-p15, 2)	1/15,000–50,000	Normal	Moderate to severe	–/+
Williams-Beuren (7q11, 2)	1/7,500–20,000	Reduced	Mild to moderate	–/+
Langer-Giedion (8q23-q24)	unknown	Normal	Mild to severe	–/+
Prader-Willi (15q11-q12)	1/10,000–30,000	Normal	Mild	–/+
Angelman (15q11-q12)	1/12,000–25,000	Normal	Severe	–/+
Miller-Dieker (17p13, 3)	1/100,000–300,000	Reduced	Profound	–/+
Smith-Magenis (17p11, 2)	1/25,000–50,000	No data	Mild to moderate	–/+
DiGeorge (10p14)	1/4,000–5,000	Reduced	Mild to moderate	+
DiGeorge (22q11, 2)	1/2,000–4,000	Reduced	Mild to moderate	+

The severity and type of the symptoms are represented from – (in cases where symptoms range from none to mild) to ++ (for those ranging from moderate to severe). The information in this table was adapted from the following resources: <http://www.orpha.net>, <http://www.nlm.nih.gov>, and <http://www.rarechromo.org>.

consultation after 13 weeks of pregnancy. The current trend focuses on the first-trimester screening (11–13 weeks), with a hormonal measurement in 9–10 weeks (bhCG + PAPP-A) and nuchal translucency and other echography parameters measurements in 11–13 weeks, plus maternal age correction (around 85–90% estimated detection rates for a 5% false positive rate for Down syndrome). Furthermore, if we add other echographic findings as nasal bone, ductus venosus, and tricuspid blood flow, the estimated detection rates reach 93–96% for a 2.5% of false positive rate for Down syndrome. Given the established effectiveness of the first-trimester screening alone, its combination with the second-trimester screening would not increase detection rates [5–7]. If this risk is higher than 1/250, invasive procedures (CV sampling or amniocentesis) are recommended [8].

Fetal chromosomal analysis has traditionally been performed using Giemsa banding (G-banding) on cultured cells in metaphase, and it is considered the gold standard detection method [9, 10]. Although the accuracy and reliability of this technique are very high, 99.4–99.8% and 97.5–99.6% for amniocentesis and CV, respectively [11, 12], the main disadvantage is that the prenatal tissue must be cultured for several days prior to analysis, whereas in conditions like abnormal ultrasound findings or where there is maternal anxiety a much quicker diagnosis would be useful. For these reasons, a rapid test to discard the presence of the most common aneuploidies (13, 18, 21, X, and Y) in live born infants is very desirable [13].

The most common rapid molecular methods for prenatal aneuploidy detection are fluorescence *in situ* hybridization (FISH) and quantitative fluorescent polymerase chain reaction (QF-PCR) [14]. These two methods allow the detection of whole chromosome aneuploidy for chromosomes 13, 18, 21,

X, and Y. Over the last three years we have introduced a new rapid prenatal diagnostic test called Prenatal-BoBs into our laboratory. This test is based on BACs-on-Beads technology, using BAC (bacterial artificial chromosome) clones attached to dyed microspheres. The advantage of this test compared to previous ones is that this multiplex assay not only includes markers for detecting aneuploidy in chromosomes 13, 18, 21, X, and Y but also contains markers for detecting nine microdeletion syndromes (DiGeorge, Williams-Beuren, Prader-Willi, Angelman, Smith-Magenis, Wolf-Hirschhorn, Cri du Chat, Langer-Giedion, and Miller-Dieker syndromes, Table 1 [15]). The microdeletion syndromes analyzed by Prenatal-BoBs were selected following these inclusion criteria: syndromes with a high relative prevalence (1/4000 to 1/200,000), significant morbidity/mortality, mild or unspecific ultrasound findings, strongly genotypic and phenotypic correlation, and deletions typically too small to be detected on a standard karyotype [15–17].

In recent years, microarray-based comparative genomic hybridization (aCGH) has been refined to determine chromosomal changes at progressively higher resolutions. In contrast to rapid aneuploidy detection, aCGH represents a comprehensive, genome-wide strategy to obtain chromosome copy-number information and, compared with conventional karyotyping, it is rapid, less labor-intensive, and readily amenable to automation. Being a genome-wide screening technique, its major advantage is that it has a vastly improved resolution compared to traditional karyotyping. The use of aCGH is recommended in concert with genetic counseling as an adjunct tool in prenatal cases where fetuses present abnormal ultrasound findings and a normal conventional karyotype, as well as in cases of fetal decease with congenital anomalies where a conventional karyotype cannot be

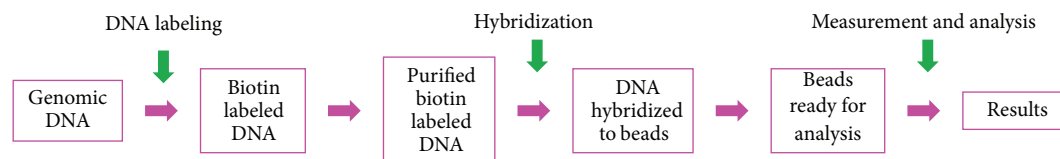


FIGURE 1: BACs-on-Beads assay flowchart.

obtained. However, its advantage is also its main handicap: high resolution allows the detection of a higher percentage of abnormalities than in a conventional karyotype but may also pick up unexpected findings and/or variants that are of unknown clinical significance [18].

In this paper we describe our experience with this new technology in prenatal diagnosis from AF and CV samples, comparing Prenatal-BoBs results with those obtained by conventional karyotypes. Furthermore, in a subset of samples, we compare Prenatal-BoBs results with the results from aCGH in cases in which the test was prescribed. The array platform employed in the present study was the CytoChip Focus (BlueGnome, UK) based on BACs technology. This array detects aneuploidies and also analyses 143 chromosomal regions of known clinical significance.

2. Materials and Methods

2.1. Human Samples for Prenatal-BoBs Analysis. Between May 2010 and June 2013, our laboratory performed 364 Prenatal-BoBs tests, 321 samples were from AF and 43 from CV samples.

Sample collection and transport to our laboratory were performed at room temperature. A minimum of 5 mL of AF was required and DNA extraction was performed immediately after reception, or alternatively the sample was gently centrifuged, the supernatant was discarded, and then pellet was stored at -20°C until extraction. CV samples were also processed immediately or stored at 4°C until DNA extraction. DNA was extracted manually from 5-4 mL of AF, or for CV samples, from a microscopically selected, entire, native villous tree or 3-5 mg of tissue, according to the manufacturer's recommendations (QIAamp DNA Mini Kit, Qiagen, Inc., Chatsworth, CA, USA). A portion of the AF or CV sample was always reserved for conventional culture and karyotyping. Furthermore, 14 samples were additionally analyzed by aCGH (BlueGnome, UK).

For conventional karyotyping, AF or CV samples were cultured for 10-12 days with 5% CO_2 at 37°C under sterile conditions. A minimum of 20 metaphase cells were analyzed with minimum resolution level of 550 bands.

2.2. BACs-on-Beads Technology. Prenatal-BoBs is a multiplex, bead-based suspension array using microspheres that are internally dyed with a combination of two spectrally distinct infrared and red fluorochromes which can produce more than 100 specific spectrums. Each bead is coupled to

DNA amplified from BACs (a total of 83 different PCR-amplified BAC clones) and analyzed using a Luminex cytometric acquisition system with two separate lasers (Luminex Corp., Austin, Texas) equipped with xPonent 3.1 software (Perkin Elmer, Turku, Finland). Experiments with acceptable quality control parameters had more than 50 beads/BACs analyzed alongside both male and female samples which were included as reference DNAs [19]. Prenatal-BoBs assesses 75 chromosomal regions involving chromosomes 13, 18, 21, X, and Y as well as the nine previously mentioned microdeletion syndromes.

Briefly, genomic DNA was extracted, labeled, purified, hybridized to BACs-on-Beads probes, bound to the reporter molecules (streptavidin-phycoerythrin), and washed. Thereafter the fluorescence signals were measured and the results analyzed (Figure 1). Once the DNA was extracted, it was amplified with a primer solution, labeled by enzymatic incorporation of biotinylated nucleotides, and purified using a PCR purification kit. Then, it was incubated overnight with BAC clones attached to dyed beads, after which the hybridized beads were transferred onto a filter plate and washed again. After washing, the beads were incubated with a reporter that binds to biotinylated DNA and then washed and resuspended for measurement according to the protocol recommended by the manufacturer.

The relative amount of DNA bound to the beads was determined using a Luminex 100/200 instrument system with xPONENT 3.1 and BoBsoft V2 analysis software that produces graphical ratio line-plots and a bar graph for each sample. A sample was defined as "duplicated/deleted" in a chromosome locus when the fluorescence in the test was higher/lower than that in the reference. Single copy gains and losses generate ratios ranging from 1.3 to 1.4 and from 0.6 to 0.8, respectively (Figure 2) [16].

2.3. Human Samples for Microarray-Based Comparative Genomic Hybridization Analysis. Approximately 7-10 mL of AF was cultured for 10-12 days with 5% CO_2 at 37°C under sterile conditions. Once a cell monolayer was obtained, it was trypsinized and DNA was extracted. DNA extraction from CV samples did not require previous cell culture. DNA was extracted using (QIAamp DNA Mini Kit, Qiagen, Inc., Chatsworth, CA, USA) according to the manufacturer's protocol and the concentration and purity of the extracted DNA were measured with a NanoDrop spectrophotometer (NanoDrop Technologies, Inc.).

Following DNA extraction, the test and reference DNA were cohybridized to the array. Briefly, 400 ng of patient

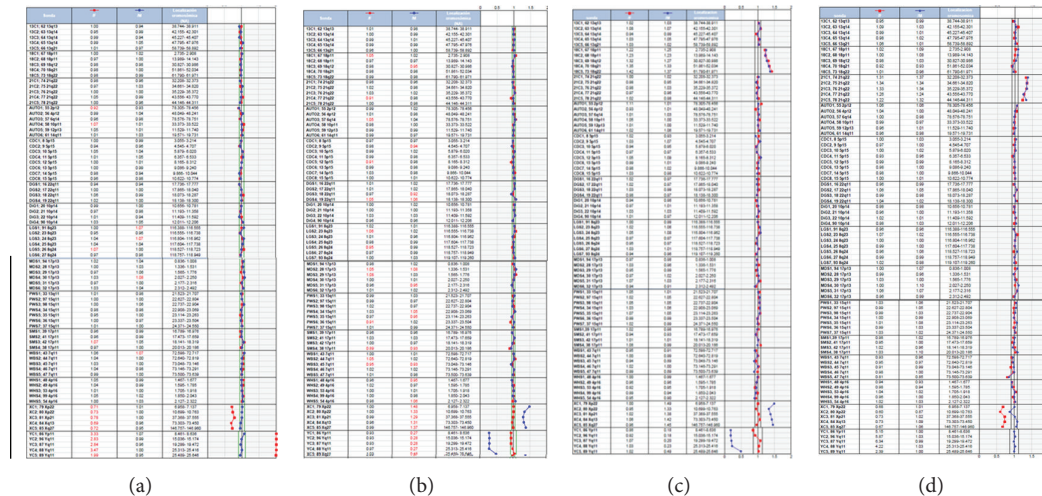


FIGURE 2: Examples of Prenatal-BoBS results: (a) male normal fetus, (b) female normal fetus, (c) female fetus with a trisomy of chromosome 18, and (d) male fetus with a trisomy of chromosome 21.

and reference DNA was labelled by random priming with Cyanine 3 and Cyanine 5 fluorescent dyes, respectively. DNA was then hybridized on the arrays (CytoChip Focus Constitutional, BlueGnome Ltd., UK) over night at 48°C. Once the arrays were washed, images were acquired using a PowerScanner microarray scanner (Tecan, Switzerland) and the image files were analyzed with the BlueFuse microarray software package (BlueGnome, UK).

3. Results

3.1. BACs-on-Beads Results. Finally, 364 Prenatal-BoBS tests were performed (321 in AF and 43 in CV samples), obtaining conclusive results in 362 cases (99.45%). Conventional G-band karyotyping was also performed and the results from cultured cells were obtained in 335 cases out of the initial 364 samples (92.03%). Karyotype failures were mainly attributed to cell culture failures, with an increased percentage of growth failure in CV samples (41.86% in CV versus 3.43% in AF samples; Fisher's exact test, $P < 0.001$).

Using Prenatal-BoBS tests, we found normal results in 309 AF (96.26%) and 35 CV (81.40%) samples. The most frequent chromosome abnormality found was trisomy 21 ($n = 13$, representing 81.25% of the total abnormal findings), but we also found an abnormal result corresponding to Cat Eye microduplication syndrome (22q11). The rate of concordance with the conventional cytogenetic method was 98.51% ($n = 335$). Three of the five samples (1.49%) that did not show concordance corresponded to chromosome abnormalities which were not detectable by Prenatal-BoBS: two Robertsonian translocations [45,XY,der(13;14)(q10;q10) and 46,XX/45,XX,der(13;14)(q10;q10)] and one reciprocal translocation [46,XY(3;4)(p22;q21)]. The other two samples correspond to polymorphisms (46,XY,15p+++ and 46,XYqh). Summarized results are shown in Table 2.

3.2. Microarray-Based Comparative Genomic Hybridization Results. Fourteen patients also underwent aCGH testing

in addition to Prenatal-BoBS and conventional G-banding karyotyping: 12 AF and 2 CV samples. In all of them, results were concordant with the karyotype and the Prenatal-BoBS test (Figure 3), including Cat Eye syndrome detected by BoBS and confirmed by CGH array platform.

4. Discussion

In this work we compared the results of a rapid aneuploidy test with those obtained with conventional karyotypes. Our results showed that Prenatal-BoBS is a reliable, robust, and efficient method for the rapid diagnosis of common aneuploidies in prenatal samples.

Despite the fact that other rapid aneuploidy tests, such as QF-PCR or FISH, have been used for several years [20], there is no consensus as to whether women at increased risk for trisomies 13, 18, or 21 should be offered stand-alone rapid aneuploidy tests or conventional G-banding karyotyping [20].

The major advantages of rapid aneuploidy tests include fast reporting (within 24 to 48 hours) and earlier anxiety relief. This fact is particularly important in the process of medical decision-making in cases of maternal anxiety, where there are fetal abnormalities found in the ultrasound examination, or if there are very few days to make a decision regarding a termination of pregnancy [21, 22]. However, about 15–30% of potential chromosome abnormalities that are detected by karyotyping would be missed using these tests in prenatal diagnosis, although this percentage is lower bearing in mind that many of those abnormalities die *in utero* [23]. It has been estimated that for approximately every 1000 amniocenteses performed, up to four potentially clinically significant chromosomal abnormalities may be missed with rapid aneuploidy tests (e.g., balanced translocations, the presence of marker chromosomes, or low grade mosaicism) [21, 22]. Therefore, mainly for this reason (loss of chromosome abnormality information in chromosomes other than 13, 18, 21, and X, and/or balanced rearrangements), clinicians

TABLE 2: Results obtained with Prenatal-BoBs—karyotype-analysis in both AF and CV samples.

	CV		AF			AF	
	Abnormal	Normal	NI	Total	Abnormal	Normal	NI Total
BoBs	7	35	1	43	11	309	1 321
Karyotype	5	20	0	25	11	299	1 310
Karyotype findings	46, XX/45, XX, der (13; 14) (q10; q10)			46, XY, 15p+++			
	0	2	0	0	3	45, XY, der (13; 14) (q10; q10)	0
		46, XY (3; 4) (p22; q21)				46, XYqh+	

CV: chorionic villus samples; AF: amniotic fluid; NI: noninformative.

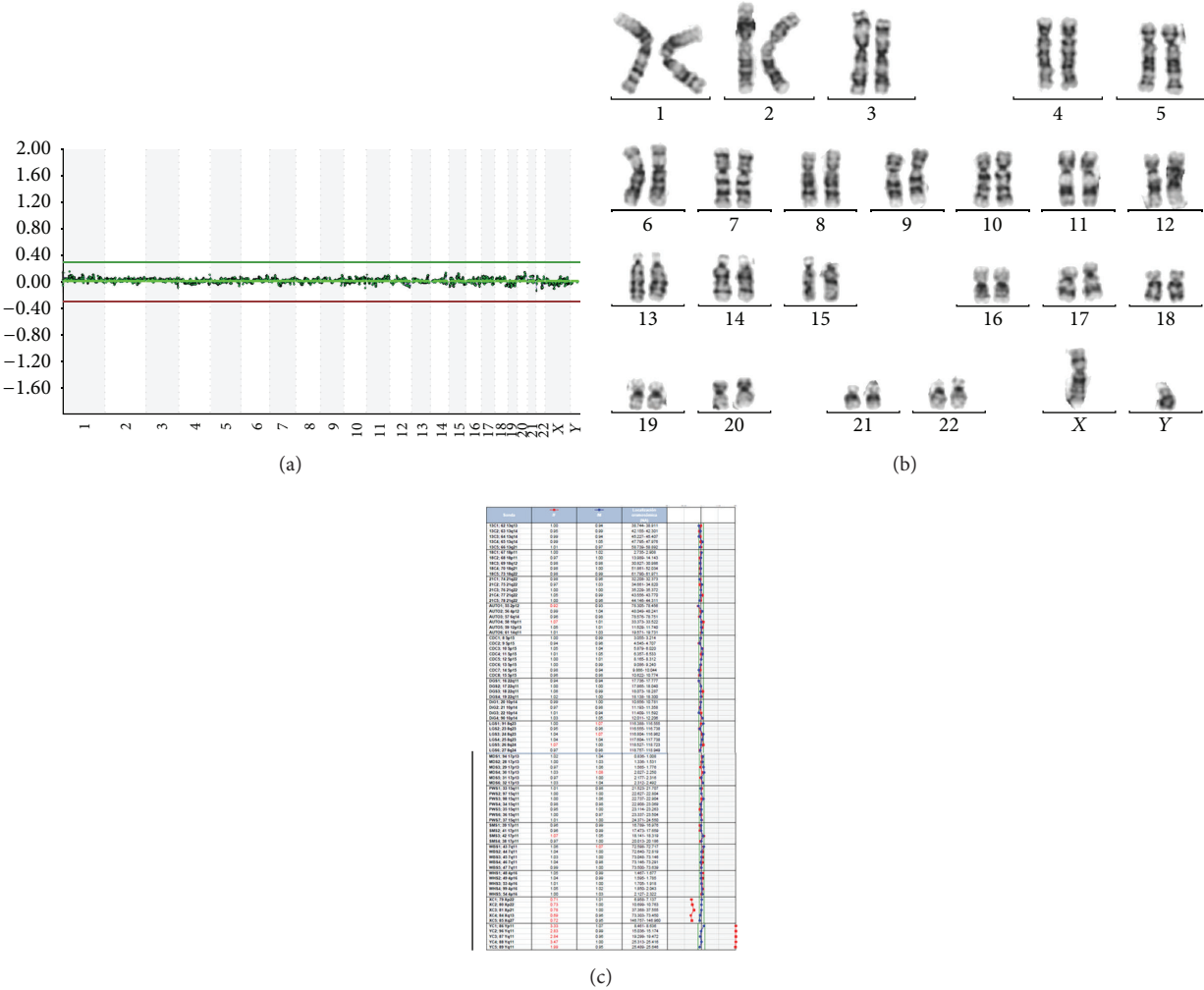


FIGURE 3: Male normal fetus: (a) CGH array focus (b), conventional karyotype, and (c) Prenatal-BoBS results.

and patients must balance the benefits and drawbacks of conventional karyotyping versus rapid prenatal tests such as Prenatal-BoBs.

Currently, there are two main trends of opinion in this issue: either rapid aneuploidy tests should replace karyotyping for indications such as positive Down's syndrome screening, in cases of advanced maternal age, and in pregnancies without ultrasound abnormalities [21–23] or both rapid aneuploidy testing and karyotyping should be carried out [20]. Those in favor of the first option generally argue that

the error rates using a rapid aneuploidy test are acceptable [24, 25] and that a final clinical decision based on full karyotyping should mostly be confined to a selected group of women with specific indications [9].

Although rapid aneuploidy tests indicate that only 1% of all invasive prenatal samples have an undetected chromosomal abnormality, a third of these can have a significant risk of serious phenotypic consequences [20], and so some authors recommend conventional karyotyping to try to avoid the potential devastating medical, emotional, and financial

consequences that an infant born with severe handicaps could have for parents and live births.

In contrast to other rapid aneuploidy tests, Prenatal-BoBs presents some special features: it is a CE-IVD certified kit with the ability to detect nine microdeletion syndromes with a high specificity (>99%; false positive rate <1%) and sensitivity (>98%; false negative rate <2%) [15, 19]. More specifically, when compared to FISH, BACs-on-Beads technology results are more objective, easier to interpret, and its protocol is robust, fast to implement in the laboratory, and amenable to automation [15, 19]. Moreover, the concordance of aneuploidies detected between karyotyping and Prenatal-BoBs was nearly 100%. An estimation of the rate of concordance for microdeletion syndromes was established in the few samples in which aCGH was prescribed and showed 100% concordance for the microdeletion syndromes included in the Prenatal-BoBs.

In the near future, the advent of massively parallel sequencing is likely to augur a big change in clinical practice in noninvasive prenatal testing (NIPT) [26]. NIPT for aneuploidy using cell-free DNA in maternal blood plasma is revolutionizing prenatal screening and diagnosis scenarios. Clinical trials have demonstrated the efficacy of NIPT for the same chromosomal abnormalities that the rapid aneuploidy testing panel analyzes [27–29]. The main advantage of this new approach is the reduction in the need for invasive prenatal diagnostic practices (such as amniocentesis) to obtain biological material from fetus, thus minimizing the risk of iatrogenic miscarriages [30, 31]. Nevertheless, this is not yet a diagnostic tool but rather a screening test, and so positive NIPT results must still be confirmed using invasive techniques.

5. Conclusions

BACs-on-Beads technology was an accurate, robust, and efficient method for the rapid diagnosis of common aneuploidies and microdeletion syndromes in prenatal samples. High concordance of detected aneuploidies was observed between karyotyping and Prenatal-BoBs test.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Male and Couple Fertility Impairment due to HPV-DNA Sperm Infection: Update on Molecular Mechanism and Clinical Impact—Systematic Review

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Recent evidences identify Human Papillomavirus (HPV) sperm infection as a possible cause of male and couple infertility. It acts through different mechanisms at various steps of human conception and early gestational development. We performed a systematic review to assess the role of HPV semen infection on male and couple infertility. Analysis of available and eligible data does not permit us to find clear evidences about clinical impact of HPV infection on fertility, although sperm parameters impairment is the most widely recognized effect. Regarding biomolecular implications, the available data are often conflicting. More studies are required to define the role of HPV sperm infection in clinical practice. The great majority of evidences are obtained by *in vitro* studies and this fact represents a limitation for the clinical management of HPV-DNA sperm infection. Understanding the biological significance of HPV-DNA semen infection could permit us to explain most of the idiopathic male and couple infertility, leading to a better management of infertile men and a better timing for sperm banking storage before ART cycles.

1. Introduction

Human Papillomavirus (HPV) infection is one of the main sexually transmitted diseases worldwide [1]. The most important clinical consequence of HPV infection is cervical cancer which remains one of the leading causes of cancer-related mortality in young and older women [2–5].

HPV infection is also related to anogenital warts and different neoplasia, such as vaginal, vulvar, penile, anal, oral cavity, head, and neck cancers [1, 4, 6]. Considering male and female, the overall prevalence of HPV infection is about 40% of population, with differences based on the HPV type and the anatomical site of infection [7].

Great progresses have been reached in understanding the pathologic mechanisms of HPV infection. Both effective screening programs (pap smears, HPV-DNA testing) and

interventions (HPV vaccination) have been developed in order to reduce HPV related disease in women [8–12].

Nevertheless few data are available on male infection [1, 13–15].

Recent findings underlined the role of HPV semen infection in male and couple infertility, focusing mainly on its high prevalence among 18–40 years old men [16, 17].

The exact localization of HPV in the spermatozoa is not well defined, even if recent studies demonstrated that the virus binds two distinct sites along the equator of the spermatozoa's head [18, 19].

Many Authors hypothesized that HPV can modify spermatic parameters causing sperm motility reduction, seminal pH alterations, and spermatozoa DNA fragmentation [18, 20–22].

Although *in vitro* studies demonstrated that spermatozoa can carry HPV-DNA and transfer it to oocytes, it is still not clear if *in vivo* the HPV-infected sperm is able to fertilize oocyte and to transfer the viral genome [17, 23–25]. These uncertainties extend to the following steps of conception because it is not clear if the infected oocytes are able to generate normal embryo and if the infection itself could interfere with implantation and subsequent pregnancy development [17].

The aim of this review is to investigate the implications of HPV sperm infection on male and couple infertility, analyzing the clinical impact on early pregnancy development and pregnancy loss, the paradigmatic spermiatic alterations, the sperm immunological modifications, and the spermatozoa HPV-related molecular changes. Finally we analysed the effect of HPV-infected sperm on fertilized oocyte, blastocyst implantation, and pregnancy development. We will also discuss the available diagnostic and therapeutic strategies, in terms of feasibility and clinical efficacy trying to make some considerations on sperm banking before ART cycles.

2. Data Sources

A Literature analysis was performed on the electronic databases Medline, Embase, ScienceDirect and the Cochrane Library, considering papers published in the time interval from 1994 to 2013.

We looked for randomized trials, observational and retrospective studies, original works, and review articles having topics as the relation between male HPV sperm infection, seminal modifications, effects on fertilized oocytes, association to apoptosis, early miscarriages, and implications on male and couple reproductive outcomes.

Key-terms included “HPV sperm infection,” “male infertility and HPV,” “sperm parameters and HPV,” “HPV infected sperm and fertilization,” “HPV and fertility outcome,” and “HPV and blastocyst apoptosis.” An accurate analysis of the references of the main works was successively performed.

We considered data from eligible studies separately, according to different topics, “clinical impact of HPV infection and fertility outcomes,” “HPV-related spermiatic modifications and their impact on fertility,” and “ability of infected semen to vehicle exogenous HPV-DNA and its impact upon ongoing pregnancy.”

3. Methods

We evaluated the clinical significance of genital HPV-DNA presence in male, female, and couple in relation to placental infection (both at term and preterm) and spontaneous miscarriage (Table 1).

The seminal parameters were defined according to WHO laboratory manual for examination and processing of human semen [5] and Hamilton Thorn motility analyser [42].

We analysed both clinical and experimental works which focused on the ability of human sperm to vehicle HPV-DNA infection into oocytes and subsequent implications (Table 2).

HPV-DNA detection in various sample tissues was based on polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) techniques.

The sperm washing techniques reported in the studies were two-layer isolate colloid wash, test-yolk buffer procedures, swim-up procedure, modified swim up with enzymatic treatment (Heparinase-III), and discontinuous Ficoll gradients. We included also studies which used DNA disc chip assay, Comet assay, and Cell Death Detection ELISA in order to detect HPV-related cells apoptosis.

Finally the hamster egg-human sperm penetration test (HEPT) has been used in some works in order to assess the ability of HPV-infected sperm to fertilize and to transfer viral genome into the oocytes.

4. Results

According to our selection criteria, only 23 works had eligible results for the aim of the review.

4.1. Clinical Impact of HPV Infection and Fertility Outcomes. Only 5 papers focused on this topic (Table 1). Hermonat et al. in 1997 firstly performed a study evaluating the presence of HPV-DNA in 25 spontaneous early miscarriages versus 15 voluntary abortions. 15 of the 25 spontaneous samples (60%) were found to be positive for HPV E6/E7 sequences versus only 3 of the 15 elective samples (20%). Semiquantitative analysis showed that the HPV detection was six-fold higher in the spontaneous abortions compared to elective ones ($P < 0.01$) [26].

Matovina et al. analyzed the products of conception from early miscarriage detecting a HPV 16–18 incidence of 7.4% [27].

Dana et al. focused on the exposure to HPV type 6/11/16/18 during pregnancy and found a spontaneous abortion rate of 6.9%, a prevalence of major birth defects of 2.2% and fetal death rate of 1.5% [28].

In 2010 Skoczynski et al. compared the prevalence of HPV infection in placentas from term deliveries and spontaneous abortions. The comparison between the two groups showed no differences in terms of HPV-DNA detection since it was found in 24.4% of placentas at term (12.8% of HPV 16/18 types) and in 17.7% of miscarriages (11.8% of HPV 16/18) [p : n.s.] [29].

Perino et al. in a cohort of 199 couples undergoing ARTs reported a prevalence of 9.5% (19/199) for HPV male infection, 17.5% (35/199) for female infection, and 4.5% (9/199) for both partners infection. After ARTs, miscarriage rate had considerably higher results in couples where a single or both partners were HPV carriers compared to noninfected couples. The comparison between the two groups resulted in 66.7% versus 15% in case of male infection ($P < 0.01$), 40% versus 13.7% in case of female infection, and 100% versus 15.9% in case of both partners infections ($P < 0.001$) [30].

The main limitations of all these studies are certainly related to the small sample size. Moreover, all the considered studies were retrospective or cross-sectional since the only perspective one was performed by Perino et al. [30].

TABLE 1: Data about studies analyzing the clinical impact of HPV infection on fertility outcomes.

Authors (Year)	Number of samples	Spontaneous aborted products: HPV +/HPV –	Electively aborted products: HPV +/HPV –	Incidence of miscarriages in pregnant exposure to HPV 6/11/16/18	Samples of placentas at term: HPV +/HPV –	Incidence of miscarriages in couple with male partner: HPV +/HPV –	Incidence of miscarriages in couple with female partner: HPV +/HPV –	Incidence of miscarriages in couple with both partner: HPV +/HPV –
Hermonat et al. (1997) [26]	40	60%/40%	20%/80%					
Matovina et al. (2004) [27]	108	7.4%/92.6%						
Dana et al. (2009) [28]	517			6.9%				
Skoczynski et al. (2011) [29]	129	17.7%/82.3% ⁺			24.4%/75.6% ⁺			
Perino et al. (2011) [30]	199 (couple)					66.7%/15% [*]	40%/13%	100%/15.9% [°]

⁺ $P < 0.05$; ^{*} $P < 0.01$; [°] $P < 0.00$.

TABLE 2: Data about studies analyzing HPV-related spermiatic modifications and their impact on fertility according to Hamilton Thorn Motility Analyzer (Data are expressed as value ± SD).

Authors (year)	Type of study	Patients	Total motility (%)	Progressive motility %	Amplitude lateral head (µm)	Percentage hyperactive (%)	Straight-line velocity (mm/sec)	Curvilinear velocity (m/s)	Average path velocity (mm/sec)	Linearity (%)
Brossfield et al. (1999) [31]	Case control study	Sperm exposed to L1 HPV-DNA n = 523	51.5 ± 0.15*	15.5 ± 0.11*	1.9 ± 0	0.5 ± 0.02	23.5 ± 0.02	43.5 ± 0.02	34.0 ± 0.04	57.0 ± 0.04
		Control sperm washed n = 498	75.0 ± 0.45*	19.0 ± 0.04*	3.1 ± 0	4.0 ± 0.09	24.5 ± 0.07	57.5 ± 0.02	41.0 ± 0	44.5 ± 0.11
		Transfected centrifuge-washed n = 114	90.0 ± 0	38.5 ± 0.61	4.1 ± 0.03	8.0 ± 0.38	31.5 ± 0.2	73.5 ± 0.05	47.5 ± 0.05	44.5 ± 0.14
		Transfected Isolate-washed n = 102	93.0 ± 0.10	33.5 ± 0.05	3.6 ± 0.12	1.0 ± 0.10	26.0 ± 0.10	56.5 ± 0.25	37.5 ± 0.25	47.0 ± 0
Connelly et al. (2001) [21]	Case control study	Transfected, TYB-washed n = 103	94.0 ± 0.40	37.0 ± 0.70	3.7 ± 0.01	4.0 ± 0	26.0 ± 0.10	58.5 ± 0.05	36.0 ± 0.20	46.5 ± 0.35
		Patients HPV-DNA 16 size = 98	48.0 ± 0.2*	5.5 ± 0.2*	1.8 ± 0*	1.0 ± 0.1		38.5 ± 0.3	28.0 ± 0.1	56.0 ± 0.2*
		Patients HPV-DNA 18 size = 80	47.5 ± 0.1*	11.0 ± 0.2*	2.7 ± 0*	1.0 ± 0.1		55.5 ± 0.7	36.0 ± 0.5	47.5 ± 0.2*
		Patients HPV-DNA 6b/11 size = 157	36.5 ± 0.1*	6.5 ± 0.1*	1.8 ± 0*	0 ± 0		31.5 ± 0.1	23.0 ± 0	57.0 ± 0.2*
		Patients HPV-DNA 31 size = 162	55.0 ± 0.5*	14.5 ± 0.1*	2.8 ± 0.1*	2.0 ± 0		45.5 ± 0.3	30.5 ± 0.1	52.5 ± 0.5*
		Patients HPV-DNA 33 size = 103	48.5 ± 0.7*	13.0 ± 0.3*	2.7 ± 0*	0 ± 0		42.5 ± 0.4	28.5 ± 0.3	52.5 ± 0.2*
Lee et al. (2002) [22]	Case control study	control subjects size = 449	74.0 ± 0.6*	17.3 ± 0.2*	3.0 ± 0.0*	2.3 ± 0.1		58.4 ± 0.2		
		Patients HPV-DNA 16 size = 433	38.4 ± 1.1*	6.0 ± 0.2*	1.3 ± 0.1*	1.4 ± 0.1		40.1 ± 0.7		
		Patients HPV-DNA 18 size = 436	56.1 ± 0.5*	9.1 ± 0.2*	2.9 ± 0.0*	2.3 ± 0.1		53.0 ± 0.2		
		Patients HPV-DNA 6/11 size = 451	47.1 ± 0.5*	7.0 ± 0.2*	2.1 ± 0.0*	3.3 ± 0.1		51.4 ± 0.4		
		Patients HPV-DNA 31 size = 434	53.4 ± 0.6*	8.6 ± 0.1*	2.7 ± 0.0*	2.4 ± 0.1		52.0 ± 0.4		
		Patients HPV-DNA 33 size = 424	46.0 ± 1.3*	10.8 ± 0.4*	3.0 ± 0.0*	2.9 ± 0.1		47.3 ± 1.3		
		controls DQA1 size = 437	47.0 ± 0.6*	6.5 ± 0.1*	2.7 ± 0.0*	4.3 ± 0.3		49.6 ± 1.0		

* P < 0.05.

4.2. HPV-Related Spermatic Modifications and Their Impact on Fertility. Only 9 eligible studies focused on this topic (Tables 2 and 3).

Both Brossfield et al. and Connelly et al. analysed the spermatic effects of HPV infection, showing that sperm cells transfected with exogenous HPV-DNA had higher percentages of total motility and progression compared to the untreated controls ($P < 0.05$) [21, 31].

On the contrary, Lee et al. demonstrated that sperm motility was reduced after HPV E6-E7 fragments expression. The percentages of progressive motility were lower in sperm exposed to all the HPV-DNA genotypes (except for the 33); the amplitude of lateral head displacement was decreased after exposure to (high-risk) hrHPV-DNA type 16 and all (low-risk) lrHPV-DNA ($P < 0.05$) [22].

In 2004 Rintala et al. analysed 65 sperm donors, 15.5% results were positive for hrHPV-DNA and 17% positive for lrHPV-DNA. They reported no HPV-related effects on sperm motility and concentration, except for pH changes [20].

Foresta et al. evaluated 200 male sperm donors observing that sperm parameters results were similar between the two groups except for the sperm motility which was significantly reduced in HPV-DNA infected men ($P < 0.05$) [18].

In the same year, Foresta et al. showed that HPV-DNA was frequently detected in exfoliated epithelial cells of lower genital tract [77.8–100%], while spermatozoa infection was detected in 72% of infertile men. Despite the mean sperm motility results significantly reduced in all infected men compared to noninfected ones ($P < 0.05$), all the remaining parameters were similar between infected or noninfected patients. A reduction of sperm concentration was registered in case of infertility, independently from HPV-DNA infection ($P < 0.05$) [32].

Considering sperm-washing procedure (in order to remove HPV-DNA from the sperm surface) Brossfield et al. showed that all the applied techniques did not positively influence the spermatic HPV-related motility and that, between them, the centrifuge wash technique results were the best ones [31].

On the other hand Foresta et al. observed that only Ficoll and swim-up procedure were useful to reduce the infection in 30% and 26% of sample, respectively ($P < 0.01$) [33].

Garolla et al. demonstrated the negative effects of HPV infection reporting that only progressive sperm motility was significantly reduced in infected semen samples ($P < 0.05$). Authors reported that direct swim-up reduces up to 24% the HPV sperm infection, while modified swim-up is able to remove completely HPV-DNA from spermatozoa ($P < 0.01$). Anyway, Authors underlined how modified swim-up results were responsible for a slight decrease of sperm motility, viability, and DNA integrity [17]. The same author in 2013, using the sperm-Mar test to detect antisperm antibodies (ASA), demonstrated that infertile men showed more frequently ASA than fertile ones ($P < 0.01$). The evidences that men with HPV-DNA infection associated to positive sperm-Mar test are affected by 24 months lower spermatozoa motility than negative ones lead the authors to propose the positive sperm-Mar test as predictor tool for future progressive spermatozoa' motility [34].

4.3. Infected Semen Ability to Transmit Exogenous HPV-DNA and Its Impact upon ongoing Pregnancy. Only 11 studies analysed sperm ability to carry exogenous HPV-DNA into oocytes and viral genome into the blastocysts and its impact in terms of fertility, implantation, and embryonic effects (Table 4).

Even if Chan et al. in 1994 showed that human sperm could be infected by HPV-DNA, Lai et al. in 1996 first assumed that spermatozoa could act like a vector for HPV transmission to sexual partners and to foetus through fertilized eggs [35, 43].

Later, both Connelly et al. and Lee et al. demonstrated increased apoptotic phenomena in sperm cells exposed to E6/E7 genes of HPV-DNA types 16 and 18 ($P < 0.05$) [21, 22].

Chan et al. demonstrated in a mouse experimental model the ability of HPV-DNA infected sperm to transmit its genome to blastocysts [37]. Cabrera et al. demonstrated the presence of HPV-DNA in both the inner cell mass and trophoblastic cells of murine infected blastocysts [23].

Foresta et al., using the hamster egg-human sperm penetration test (HEPT), demonstrated the sperm ability to transfer both the capsid protein L1 and E6/E7 viral genes to oocytes with a subsequent gene expression by transfected blastocysts [25].

Calinisan et al., transfecting blastocysts with the E6-E7 region of types 16, 18, 31, and 33, detected the presence of DNA fragmentation only in the subgroup of blastocysts infected by HPV-DNA type 16 ($P < 0.05$). The ability of HPV-DNA type 16 to induce DNA fragmentation and subsequent trophoblastic death was also confirmed by You et al. in 2002 [38].

Hennemberg et al. confirmed the direct HPV16 inhibitory effect on blastocysts growth only at the two-cell embryo stages but not on the 4–8 cells ones [40].

Gomez et al. showed that the apoptosis rate in transfected trophoblastic cells was 3-fold (2.4–3.7) and 5.8-fold (5.6–5.9) greater at 3 and 12 days, respectively, if compared to negative controls ($P < 0.01$). Simultaneously, authors reported that the invasion ability of transfected trophoblast progressively decreased from day 3 to day 15 (25.2–57.6% lower than negative controls) ($P < 0.001$) [41].

The limitations of all these studies were certainly related to the artificial conditions linked to the *in vitro* experiments and murine models which may not reflect the real *in vivo* human situations. All these data should be validated by large *in vivo* perspective studies but unfortunately the literature lacks information about this field because of ethical policies concerning human reproduction experiments.

5. Discussion

It is widely proven that sexually transmitted infections (STD) represent a possible cause of male infertility since they can induce urethral stickiness, epididymis inflammation, and orchitis up to testicular failure [44]. The impairment of sperm motility and DNA integrity through an autoimmune mechanism could result in obstetric complications such as early miscarriages and preterm deliveries [27, 45].

TABLE 3: Data about studies analyzing HPV-related spermiatic modifications and their impact on fertility according to WHO laboratory manual for the examination and processing of human semen (Data are expressed as value \pm SD).

Authors (year)	Study type	Patients	Sperm concentration (10 ⁶ /mL)	Semen volume (mL)	Total sperm count (10 ⁶)	pH	Progressive motility %	Normal morphology %	Viability %
Rintala et al. (2004) [20]	Case control	High-risk HPV DNA (+) $n = 10$ High-risk HPV DNA (-) $n = 55$		3.07 4.03		7.37 7.51	54.2 56.5		65.2 69.6
Foresta et al. (2010a) [18]	Cross-sectional clinical	Sexually active subjects HPV (+) $n = 10$ Sexually active subjects HPV (-) $n = 90$ Virgin subjects $n = 100$	57.5 \pm 30.4 60.2 \pm 31.0 58.3 \pm 29.1	2.9 \pm 1.6 2.4 \pm 1.6 2.7 \pm 1.5	174.3 \pm 115.8 175.8 \pm 154.5 174.5 \pm 164.7	7.7 \pm 0.3 7.6 \pm 0.2 7.6 \pm 0.3	37.7 \pm 16.8* 53.7 \pm 18.2* 53.7 \pm 19.0*	31.5 \pm 8 33.1 \pm 11.1 32.8 \pm 10.6	83.5 \pm 7.9 84.6 \pm 8.6 83.6 \pm 7.6
Foresta et al. (2010b) [32]	Cross-sectional clinical	Patients with genital warts HPV (+) $n = 14$ Patients with genital warts HPV (-) $n = 12$ Subjects with HPV + partner HPV (+) $n = 27$ Subjects with HPV + partner HPV (-) $n = 39$ Infertile patients HPV (+) $n = 11$ Infertile patients HPV (-) $n = 97$ Fertile controls HPV (+) $n = 2$ Fertile controls HPV (-) $n = 88$	53.5 \pm 30.0* 56.2 \pm 33.8* 48.5 \pm 23.0* 50.1 \pm 22.3* 30.0 \pm 21.5* 35.2 \pm 23.0* 60.5 \pm 31.5* 58.7 \pm 30.8*	2.6 \pm 1.7 0.8 \pm 1.8 2.8 \pm 1.2 2.5 \pm 1.3 2.9 \pm 1.9 3.0 \pm 1.5 2.5 \pm 1.6 2.6 \pm 1.6	167.6 \pm 111.7 177.1 \pm 126.4 172.8 \pm 110.2 178.4 \pm 102.3 99.4 \pm 88.8 102.9 \pm 100.9 175.5 \pm 131.6 176.0 \pm 139.6	7.7 \pm 0.2 7.4 \pm 0.3 7.6 \pm 0.2 7.7 \pm 0.4 7.7 \pm 0.3 7.6 \pm 0.3 7.6 \pm 0.2 7.7 \pm 0.2	36.2 \pm 18.7* 56.2 \pm 19.8* 38.4 \pm 13.2* 53.8 \pm 16.5* 33.9 \pm 15.9* 51.7 \pm 16.2* 55.5 \pm 17.6* 54.2 \pm 17.9*	32.6 \pm 10.7 36.3 \pm 14.4 31.8 \pm 11.2 31.8 \pm 11.2 32.9 \pm 13.9 33.1 \pm 11.1 33.5 \pm 10.6 33.0 \pm 13.5	80.2 \pm 9.1 81.3 \pm 10.5 82.4 \pm 8.8 82.4 \pm 8.8 79.8 \pm 8.6 84.6 \pm 10.7 81.7 \pm 9.4 83.9 \pm 8.0
Foresta et al. (2011b) [33]	Cross-sectional clinical	Infected infertile patients $n = 32$ HPV-infected patients $n = 22$ Control subject $n = 13$ LI-incubated sperm (pool)	32.4 \pm 21.1 29.0 \pm 10.3 30.5 \pm 9.8	3.0 \pm 1.1 3.1 \pm 0.9 3.3 \pm 1.0	100.2 \pm 73.4 87.7 \pm 36.3 98.8 \pm 46.7	7.6 \pm 0.3 7.6 \pm 0.2 7.5 \pm 0.3	29.7 \pm 13.8* 29.6 \pm 14.2* 42.4 \pm 22.7*	17.8 \pm 9.1 19.0 \pm 6.3 21.1 \pm 7.5	78.3 \pm 11.6 81.3 \pm 6.3 83.8 \pm 8.3
Garolla et al. (2012) [17]	Case-control						22.6 \pm 8.7*	20.9 \pm 6.5	82.8 \pm 8.7
Garolla et al. (2013) [34]	Cross-sectional clinical	Infertile HPV-infected patients $n = 61$ Infertile noninfected patients $n = 104$ Control subjects $n = 92$	32.0 \pm 11.2* 34.6 \pm 9.8* 51.3 \pm 8.4*		94.2 \pm 36.5* 108.8 \pm 44.5* 156.0 \pm 42.9*		29.0 \pm 11.4* 47.8 \pm 11.0* 53.4 \pm 11.4*	18.8 \pm 6.2 18.5 \pm 4.3 21.3 \pm 4.7	80.0 \pm 7.1 83.2 \pm 5.1 83.6 \pm 5.1

* $P < 0.01$; * $P < 0.05$.

TABLE 4: Data about studies analyzing exogenous sperm HPV-DNA vehicle and its impact on product of conception.

Authors (year)	Number of samples	Aim of the study	Study setting	Main outcomes	Conclusions
Chan et al. (1994) [35]	42	Detect presence of HPV-DNA 16/18 in sperm cells	HPV-DNA detection trough PCR	HPV-DNA detection (PCR): HPV 16: 64.3% HPV 18: 38.1% HPV L1: 35.7%	(i) The study demonstrated the presence of HPV-DNA in sperm cells (ii) The result suggest a possible role of sperm as a vector for HPV
Lai et al. (1997) [36]	24	Detect presence of HPV-DNA 16/18 in both seminal plasma and sperm cells	HPV-DNA detection trough PCR	HPV-DNA detection (PCR): HPV 16: 33.3% (8.3% seminal plasma; 25% sperm cells) HPV 18: 79.1% (33.3% seminal plasma, 45.8% sperm cells)	(i) HPV can infect both sperm cell and seminal plasma (ii) HPV-DNA 16 and 18 are expressed actively in infected sperm cells (iii) The HPV infected sperm cells can behave as vectors for the transmission of HPV to fetuses through fertilized eggs
Chan et al. (1996) [37]	—	Demonstrate that sperm cells transfected with HPV-DNA 16/18 can vehicle viral genome to cells of uterus and embryo	(i) Non-human (mouse) experimental study (ii) HPV-DNA detection trough PCR	(i) Blastocyst took-up HPV-DNA fragments from both HPV-DNA 16 and HPV-DNA 18 of carrier sperm (ii) The sperm cells transferred HPV-DNA18 but not 16 fragments to the uterine cells at both the cervical-uterine end and the uterine-tubal end	(i) It is possible to apply the transmission of HPV DNA from the sperm to the embryos and cells of the reproductive tract (ii) The study suggests sperm as a vector for the transmission of DNA to the developing embryo
Cabrera et al. (1997)	—	Demonstrate that exogenous HPV-DNA taken into blastocysts is localized to both the inner cell mass and trophoblast cells	(i) Non-human (mouse) experimental study (ii) Mouse blastocysts exposed to migrating human sperm cells carrying exogenous DNA fragments from HPV 16 and 18 (iii) HPV-DNA detection trough PCR	Mouse blastocysts transfected by carrier sperm with HPV-DNA 16 and 18 showed localization of the HPV-DNA to both the inner cell mass and trophoblast cells	(i) Exogenous DNA taken into blastocysts is localized to both the inner cell mass and trophoblast cells (ii) Only live sperm exhibited the capacity to carry various sizes of exogenous DNA, suggesting the involvement of active cell membrane mechanism in the transference process
Lee et al. (2001)	8	Demonstrate that HPV-DNA can induce sperm cell apoptosis through the determination of p53 exons 5 and 8 integrity	Sperm apoptosis was detected trough Comet assay	Sperm apoptosis (Integrity of exons 5 and 8 of the p53 gene) Exon 5 ratio HPV 16 1.35 ± 0.38 HPV 18 0.86 ± 0.05 HPV 6b/11 1.26 ± 0.21 HPV 31 0.91 ± 0.20 HPV 33 0.98 ± 0.06 Percentage of sperm apoptosis density: pixels, mean ± SEM): Control (DQAI) 167.3 ± 2.4 HPV 16 125.9 ± 3.8 HPV 18 163.2 ± 3.3 HPV 31 154.0 ± 3.5 HPV 33 166.7 ± 3.7 HPV 6/11 168.0 ± 2.8	The data suggest that different HPV types preferentially degrade different exons of important genes
Connelly et al. (2001) [21]	6	Demonstrate that HPV-DNA can induce sperm cell apoptosis	Sperm apoptosis was detected trough DNA disc chip assay		(i) HPV types 16 and 31 might lead to failed embryonic development through sperm apoptosis

TABLE 4: Continued.

Authors (year)	Number of samples	Aim of the study	Study setting	Main outcomes	Conclusions
You et al. (2002) [38]	—	(i) Determine if the DNA of trophoblast were disrupted by the presence of HPV DNA	(i) Use of recombinant adeno-associated viruses (rAAV) to introduce the HPV-16 E6 and E7 oncogenes into trophoblasts	HPV-16 oncogene expression may lead to outright trophoblast death	(i) These changes to trophoblast might be responsible of trophoblast and placental alteration and could contribute to spontaneous abortions
Calinisan et al. (2002) [39]	—	(i) Determine if the DNA of blastocysts was disrupted by the presence of HPV DNA (ii) Determine if the intensity of DNA damage was associated with the type of HPV	(i) Non-human (mouse) experimental study (ii) Blastocysts infected with HPV-DNA 16, 18, 31, or 33 (iii) Blastocyst apoptosis was detected through Comet assay	Mouse blastocysts apoptosis (DNA fragmentation) (mean pixel intensity ± 1 SD) [§] HPV 16: 190.1 ± 24.4 ⁺ HPV 18: 180.1 ± 21.7 ⁺ HPV 31: 171.9 ± 33.2 ⁺ HPV 33: 179.5 ± 12.0 ⁺	(i) Only HPV-DNA 16 was associated with significant DNA fragmentation (ii) The intensity of DNA damage was not linked to the specific type of HPV
Henneberg et al. (2006) [40]	—	(i) Assess the development of early embryos exposed to HPV DNA (ii) Analyze the blastocyst hatching process after HPV exposure.	(i) Non-human (mouse) experimental study (ii) Two-cell and 4–8-cell mouse embryos exposure to HPV-DNA 16 and 18	(i) HPV 16 and 18 inhibited two-cell embryo development but not 4–8-cell stage (ii) 25.9% less blastocyst formed with HPV 16 exposure (iii) 25.9–31.8% more degenerated embryos with HPV 16 exposure	(i) Demonstration of HPV embryo stage-specific effects on early development (ii) HPV 16 was shown to decrease blastocyst formation (iii) HPV 18 inhibited the blastocyst hatching process
Gomez et al. (2008) [41]	—	(i) Determine if HPV infection of extravillous trophoblast cells reduces cell invasion and induce apoptosis (ii) Determine if placental infection is associated with adverse reproductive outcomes attributed to placental dysfunction	(i) Extravillous trophoblast cells transfected with the HPV-16 genome were detected (ii) Apoptosis assay (Cell Death Detection ELISA) (iii) Invasion assays (Cell Invasion Assay Kit)	(i) Rates of apoptosis were 3- to 6- fold greater in transfected cells than in non-transfected cells [†] (ii) Invasion of transfected cells through extracellular matrices was 25–58% lower than that of the controls [‡]	(i) HPV extravillous trophoblast infection induces cell death and may reduce placental invasion into the uterine wall (ii) HPV infection may cause placental dysfunction and could be associated with adverse pregnancy outcomes, (such as preterm delivery)
Foresta et al. (2011a) [25]	—	(i) HPV localization in sperm cell (ii) Demonstrate that HPV-DNA transfected sperm cells can transfer viral genome into oocytes	(i) Fluorescence in situ hybridization for HPV (FISH) (ii) Hamster Egg Penetration Test (HEPT) with human sperm transfected with HPV E6/E7 plasmid	(i) HPV is localized at the equatorial region of sperm head through interaction between the HPV capsid protein L1 and syndecan-1 (ii) HPV transfected sperms are able to penetrate the oocyte (iii) Viral genes are then activated and transcribed into the oocyte	(i) Sperm might function as vectors for HPV transfer into the oocytes

* Lower head intensity (in pixels) represented greater DNA fragmentation. (The data were expressed as mean \pm SEM) [§] Lower pixel intensity is associated with more DNA fragmentation.

⁺ $P < 0.05$; [†] $P < 0.01$; [‡] $P < 0.001$.

The high prevalence of male HPV detection raised strong interest for its possible consequences on male fertility. HPV infection natural history in men is still mostly unknown. Recent findings suggest that the incidence of genital HPV infection is 38.4 per 1000 male/months (95% CI 34.3–43.0). The mean duration of male infection results of 7–52 months are (6.80–8.61) for any HPV type and of 12–19 months (7.16–18.17) are for hrHPV 16 [46].

5.1. Clinical Impact of HPV Infection and Fertility Outcomes.

All *in vitro* studies showed a negative influence of HPV upon several aspects of male fertility. However, only few studies investigated the *in vivo* effects of HPV on human reproduction phases. In 1997 Hermonat et al. reported the correlation between HPV infection and spontaneous miscarriages in first trimester of pregnancy. The higher percentage of HPV-DNA detection, if compared to voluntary abortions, paved the way to consider HPV as one of the possible etiologic agents responsible for early pregnancy loss [26]. This hypothesis was not confirmed by Matovina et al. who found HPV-DNA in only 7.4% of spontaneous miscarriage specimens. Anyway the authors did not exclude the possibility of HPV-DNA trans-placental transfer [27].

Nowadays, in spontaneous conception, the rate of early miscarriages and major birth defects does not seem greater in HPV-exposed couples than in unexposed ones [28, 29]. However, considering *in vitro* fertilization techniques, this aspect seems to be of crucial importance. The first prospective study on this topic reported a significant pregnancy loss increase in couples undergone ARTs and male partner with semen HPV infection (miscarriage rate of 100% when both partners results are infected) [30].

So we can conclude that the role of HPV infection in spontaneous abortion is still not clear and its association with adverse pregnancy outcomes is not certainly demonstrated. Further perspective longitudinal studies are necessary to better understand the possible roles of HPV infection in early miscarriage and in other adverse pregnancy outcomes.

However, if the evidences of Perino et al. will be confirmed, a major care on HPV status of couples attempting ART procedures should be necessary. Thus, HPV male vaccination could represent a possible strategy for male fertility preservation and for ART success rate improvement.

5.2. HPV-Related Spermatic Modifications and Their Impact on Fertility.

The molecular mechanisms by which HPV could impair sperm quality and fertilized oocytes development has been neither completely demonstrated nor clarified.

From our analysis, HPV presence in spermatozoa may be associated with an impairment of sperm parameters. Lai et al. firstly demonstrated that HPV presence in sperm could affect spermatozoa's motility (lower velocity, straight-line velocity, and mean amplitude of lateral head displacement) although it seems to be not statistically significant. A possible association between HPV semen infection and asthenozoospermia has also been described [36]. These findings seem to be partially confirmed even by following studies. Previous works showed

that prewashed sperm specimens transfected with L1 HPV-DNA fragments had an increased motility and progression but they did not elucidate the possible mechanisms [21, 31]. On the other hand, Lee et al. described a possible association between HPV-DNA presence, sperm motility reduction, and total progression after 24 hours of incubation [22].

The slight motility increase described by Connelly et al. in HPV exposed spermatozoa might be explained considering that the observation was performed after two hours of incubation. This fact suggests that HPV-DNA requires a suitable interval time to determine molecular changes on sperm motility apparatus. The authors reported a mean reduction of lateral head amplitude even if the virus seems not able to decrease oocytes sperm fertilization ability [21].

Rintala et al. in HPV positive semen detected only a pH change without any impairment of other parameters [20].

For many years semen pH impairment was considered the most important mechanism explaining fertility decrease due to asymptomatic genital infections, especially the bacterial ones.

In our opinion pH impairment represents only one of the several factors that may influence male fertility, since the aetiological mechanism could involve many other factors such as mean sperm motility, presence of ASA on the spermatozoa surface, and qualitative semen parameters impairment till asthenozoospermia [18, 32–34].

Since several cases of reported idiopathic asthenozoospermia do not present any known risk factor except for the positivity to HPV-DNA, semen washing procedures could represent a way to improve spermatozoa quality before ART procedures [18, 32, 34].

Starting from these considerations, many studies proposed different semen washing methods to eliminate HPV-DNA sperm infection, but all proposed techniques failed in the scope, except for the modified swim-up technique. This last one contemplates enzymatic treatment (Heparinase-III), apparently able to completely remove HPV-DNA from sperm cells although with a deterioration in semen quality.

Nowadays all the available data suggest that the classic procedures cannot eliminate HPV sperm infection and that HPV-DNA semen screening can be considered only as an epidemiological investigation helping to define the best timing (regression of semen infection) to start ART cycles.

It is well demonstrated both in men and women that HPV infection is generally transient and only few patients are subject to persistent infection. Our suggestion to test the sperm for HPV before sperm banking and ART cycles could represent the last option for the clinicians to try to improve the semen quality both for fresh use and for frozen preservation. Certainly, the vaccine option could have useful results for both male and female patients presenting poor reproductive outcomes till the couple infertility [47].

5.3. Ability of Infected Semen to Vehicle Exogenous HPV-DNA and Its Impact on Pregnancy Evolution.

The HPV-DNA presence in sperm and the related modification induced by the infection seem to play a role in physiopathology of unexplained male infertility. However, *in vitro* evidences

showed that HPV infected spermatozoa maintains the ability to fertilize oocytes and to express viral genome in the product of conception. This suspect seems to be confirmed by a higher rate of *in vitro* blastocysts and trophoblastic HPV-related apoptosis probably responsible of *in vivo* fertility rate reduction of infected couples.

Evidences reported by Chan et al. [35], Lai et al. [43], Pérez-Andino et al. [19], and Foresta et al. [25] clarified many aspects of the overall fertility reduction in couple with male HPV infection. The HPV ability to bind the spermatozoa in two distinct sites of the equatorial region through sydecin-1 (a proteoglycan expressed almost exclusively in the equatorial region of sperm head) can explain the sperm ability to carry viral genome into fertilized oocytes and blastocysts [19, 21, 22, 25].

In both experimental murine and *in vivo* human models it was found that HPV genomes are expressed in fertilized oocytes, blastocysts, and trophoblastic cells [23, 25, 37]. The viral genome could induce cellular changes such as inhibition of zygote growth, decrease in blastocyst formation, inhibition of blastocyst hatching process, and DNA fragmentation and apoptosis, and thus results are often lethal for early embryo development [38–40].

In vitro experiments showed that HPV transfected blastocysts and trophoblastic cells were affected by a reduction in decidua invasion capacity, potentially responsible for a failure of maternal uterine wall invasion by the extravillous trophoblastic cells, subsequent placental dysfunction, and adverse pregnancy outcomes (i.e., early miscarriage) [41].

Nowadays both the exact mechanism and timing through which the HPV infection modifies trophoblastic genes expression and increases cell death have not yet been understood. All the considered studies have important limits which should not be underestimated, linked to the *in vitro* artificial and experimental conditions. Further validation in *in vivo* studies could be therefore needed.

5.4. New Insight: HPV Male Vaccination and Sperm Effects. The actual high prevalence of HPV sperm infection represents a large scale problem for the sperm donors banks [48, 49].

HPV screening for all semen samples before sperm banking should be considered as a real option of semen storage for future ART cycles.

The rationale is that, when possible, the semen banking should be postponed until HPV infection resolution. The main limitations in this field are linked to the poor knowledge of HPV male infection natural history and of the most adequate interval time to postpone the storage.

Moreover, neither evidences are available about long term effects of previous HPV sperm infection nor which parameters could recover after the infection-linked damages.

In absence of effective and safety sperm washing procedures able to eliminate the infection, HPV male vaccination should be considered as a possible strategy for the prevention of HPV semen impairment and for the improvement of couple fertility outcomes [4, 30, 46, 48, 50, 51].

Male vaccination could represent also a reliable option for couples undergoing ART cycles because fertility of female partner results yet partially compromised: this could solve the problem linked to sperm banking, avoiding the potential HPV negative effect on sperm quality [52].

The biggest concern of Public Health Programs and clinical practitioners about the cost-effectiveness of HPV male vaccination is linked to the several biases affecting available data. For example, a recent study by Kim and Goldie concluded that the HPV vaccination of all 12-year-old boys would not be cost effective [53]. All readers should realize that this conclusion was obtained despite strong bias: oncologic safety and efficacy in men were not included since it considered only women outcomes; only heterosexual couples were considered without including outcomes in homosexual men; it did not evaluate data about incidence, mortality, and quality of life linked to cancers different from cervix and, finally, it did not contemplate the possible role of HPV male infection on fertility.

6. Conclusions

Most of analysed data suggested that the HPV sperm infection could be responsible for a decreased fertility rate through different mechanisms acting at various steps of the human embryo development.

The still debated clinical features related to HPV-DNA sperm infection are the increased risk of early miscarriage and the higher incidence of unexplained male infertility (related to sperm parameters impairment). HPV-DNA sperm test should be realized in semen donors and before ART cycles, despite the limitations linked to *in vitro* studies evidences. Improvement in the knowledge of HPV-DNA sperm infection mechanisms, timing, and link to fertility impairment could explain most of the actual “idiopathic” male and couple infertility.

The cost-effectiveness analysis related to fertility improvement in HPV vaccinated male requires further evaluation in the next future.

The implications related to possible achievement of herd immunity after a mass population vaccination program could overcome the existing doubts, explaining and resolving the unclear aspects in this field.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

New Advances of Preimplantation and Prenatal Genetic Screening and Noninvasive Testing as a Potential Predictor of Health Status of Babies

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The current morphologically based selection of human embryos for transfer cannot detect chromosome aneuploidies. So far, only biopsy techniques have been able to screen for chromosomal aneuploidies in the in vitro fertilization (IVF) embryos. Preimplantation genetic diagnosis (PGD) or screening (PGS) involves the biopsy of oocyte polar bodies or embryonic cells and has become a routine clinical procedure in many IVF clinics worldwide, including recent development of comprehensive chromosome screening of all 23 pairs of chromosomes by microarrays for aneuploidy screening. The routine preimplantation and prenatal genetic diagnosis (PND) require testing in an aggressive manner. These procedures may be invasive to the growing embryo and fetus and potentially could compromise the clinical outcome. Therefore the aim of this review is to summarize not only the new knowledge on preimplantation and prenatal genetic diagnosis in humans, but also on the development of potential noninvasive embryo and fetal testing that might play an important role in the future.

1. Introduction

A quarter of century has already passed since the first application of preimplantation genetic diagnosis (PGD) by Handy-side in 1990 [1]. In the beginning, this method was applied for monogenic diseases and sex-linked disorders. Later, the major indications expanded for detection of chromosomal abnormalities and presence of translocations in either partner. The recent development of comprehensive chromosome screening of all 23 pairs of chromosomes by microarrays or the so-called preimplantation genetic screening (PGS) for aneuploidies and translocation in human embryos was achieved [2]. In the last decade, the PGD list was expanded for other purposes such as cancer predisposition disorders, rhesus incompatibility, mitochondrial disorders, and human leukocyte antigen typing [3–8]. Nowadays, PGD could be offered for any disorder for which molecular testing can be performed. The point of this technique is the removal of cells from the preimplantation embryos in the IVF program, genetic testing of these cells, and replacement (or freezing) of normal embryos into the uterus. In spite of the great

advantage of PGD and its benefit for couples with genetic disorders (birth of a healthy baby or prevention of repeated spontaneous abortion), the embryo biopsy is an aggressive method, which may disturb the embryo. New approaches are being developed for indirect evaluation of the genetic status of human embryos in the IVF programs. The aim of this review is to summarize the recent knowledge on preimplantation (PGD) and prenatal genetic diagnosis (PND) and the potential use of noninvasive testing of embryos and fetuses in the future.

2. Preimplantation Genetic Diagnosis

PGD or preimplantation genetic screening (PGS) is performed at three different stages of the embryo development: (1) oocyte polar body biopsy [9] before and after fertilization, (2) blastomere biopsy [10] at cleavage stage (Figure 1), and (3) trophoctoderm (TE) tissue biopsy at blastocyst stage [11]. There are certain pitfalls related to the genetic diagnosis of single cells such as amplification failure, preferential amplification, allele dropout (ADO), and contamination with

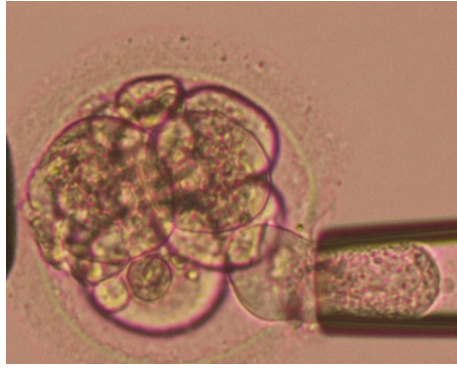


FIGURE 1: Preimplantation embryo biopsy in the in vitro fertilization program. Aspiration of a blastomere into the biopsy pipette.

extraneous DNA [12, 13]. Polar body or blastomere cells are more prone to these problems since they contain a limited amount of material available for genetic analysis. However, trophectoderm tissue biopsy at the blastocyst stage has the advantage of removing more cells (5–10), which potentially reduces the occurrence of these risks [11]. In addition to the technical advantages, blastocyst stage biopsy has the advantage of selecting developmentally more competent embryos for diagnosis that could improve pregnancy rates while at the same time it decreases the cost of the PGD study [14].

3. Oocyte Polar Body Biopsy

Biopsies of the first and second polar bodies have been performed for three decades [16], but nowadays only several countries use this technique routinely. The first polar body biopsy is applicable for couples with ethical concerns as preconception genetic diagnostic tool [17]. Another stage for biopsy is the period before syngamy (and after an ICSI procedure). The laws in several countries (e.g., Austria, Switzerland, and Germany) establish this procedure because it prohibits the genetic testing of cells derived from cleavage preimplantation embryo. Worldwide there are only few PGD laboratories where the genetic testing of the first and second polar bodies is still routinely used (e.g., RGI, Chicago, USA). Today this method is not a common practice. This method has more disadvantages than advantages such as lack of information for aneuploidies of paternal and mitotic origin; need of analysis of a huge amount of polar bodies and therefore unnecessary work and kits for diagnostic (some of the oocytes will not be fertilized and some of the zygotes will not reach the blastocyst stage); being highly expensive; chance for aneuploidy compensation (2–4%) [18]; high risk of aneuploidy (32.5%) [19]. Some advantages of this “early biopsy” are the diagnostics of oocytes themselves and female infertility, lack of mosaicism, and the minimal risk of affecting the embryo during the biopsy. However, there are still some perspectives, which could be used in reproductive medicine. The polar body genetic analysis might be an interesting approach—the aim is to select the oocytes after in vitro maturation procedure, which are appropriate for in vitro

fertilization, and to improve the outcome of oocyte in vitro maturation in the clinical practice.

4. Preimplantation Embryo Biopsy

This is a biopsy for the later stages—the cleavage stage or the blastocyst stage embryo [11, 20]. However, day 3 embryo biopsy still possesses a high risk of mosaicism: from 55% to 73% [21–23]. In general, blastomere biopsy has limitations because of the fact that up to 60% of embryos at cleavage stage of development exhibit mosaicism, where at least one cell has a different ploidy from other cells in the embryo [24, 25]. Additionally, many cleavage stage embryos diagnosed as aneuploid with blastomere biopsy will “self-correct” by blastocyst stage, which, from a clinical stand point, may decrease the chances of a live birth by prematurely labeling an embryo as abnormal [26–30]. Even though, blastomere biopsies often successfully predict ploidy of the fetus, limitations such as mosaicism and self-correction complicate the issuing of a correct diagnosis, even when using highly accurate PGS technologies.

Mosaicism occurs also in blastocysts, but apparently at lower levels than in cleavage stage embryos. In a study of Johnson et al. [25] the rate of mosaicism between inner cell mass (ICM) and trophectoderm (TE), as well as between TE fractions, was only 3.9%. In addition, it was evident that the aneuploidy rate is significantly lower (38.8%) in blastocysts than in embryos at earlier stages (51%) [20]. Cleavage stage PGD/PGS could have negative impact on clinical outcome due to the embryo biopsy procedure whereas day 5 diagnosis (and freezing the biopsied embryos) allows the biopsy of cells that are not involved in the formation of the embryo rather than cells that may be committed to forming the ICM [11]. In addition to all these facts, the optimized uterine environment in the next cycle and the possibility of a single embryo transfer (SET) are in favor of day 5 embryo biopsy and genetic analysis. According to different studies this strategy may culminate in a pregnancy rate per transfer of 63% to 70.5% [31, 32].

5. Correlation between Genetic and Indirect Methods of Embryo Selection: Noninvasive Preimplantation Genetic Testing without Embryo Biopsy in the Future?

Is it possible for noninvasive preimplantation diagnosis to exist in the future as a unique tool? There is a tendency for noninvasive screening and searching correlations between different quality parameters of gametes, zygotes, embryos (vacuoles in sperm heads, spindle position in mature oocytes, cleavage intervals of zygote, and embryo developmental dynamics) and aneuploidy rates in human gametes and embryos. Different methods have been suggested for this approach.

5.1. Selection of Sperm for Fertilization. The intracytoplasmic morphologically selected sperm injection (IMSI) enables the selection of sperm for fertilization and improves poor embryo

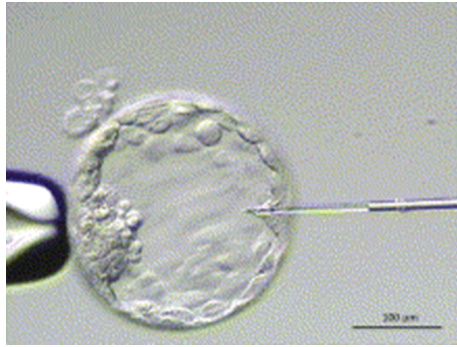


FIGURE 2: Aspiration of the blastocoele fluid using the ICSI pipette (source: [15]).

development in couples with poor semen quality. A randomized study of the team of Virant-Klun [33] showed that the IMSI procedure improves embryo development along with the laboratory and clinical outcomes of sperm microinjection in the same infertile couples with male infertility and poor embryo development over the previous ICSI attempts. Some studies have already confirmed that there is an increased aneuploidy rate in spermatozoa with large vacuoles in their heads [34]. The analysis of sperm, performed after morphological selection by high-magnification microscopy, indeed showed a significantly better mitochondrial function, chromatin status, and euploidy rate than observed in unselected cells. Moreover, a recent study showed that chromosomal architecture might be disturbed in spermatozoa with large vacuoles in their heads [35]. Therefore, it could be speculated that the selection of good-quality sperm could decrease the aneuploidy rates in the resulting embryos. As the technique seems noneffective for any unselected patients, relevant indications for the use of IMSI need to be defined. For patients with severe male factor evidence suggests higher clinical pregnancy and lower miscarriage rates [36]. In addition, it is known that the presence of all 23 pairs of chromosomes is a prerequisite for normal implantation and healthy fetal development in humans. The improved outcome of in vitro fertilization using IMSI was also observed in patients with teratozoospermia due to improved development and quality of embryos [37].

5.2. Blastocoele Fluid and DNA Extraction without Biopsy.

The analysis of the fluid from the blastocyst cavity (blastocoele) is an interesting approach. Using the real-time PCR, the study showed [15] that genomic DNA was present in about 90% of blastocoele fluid samples harvested during the vitrification procedure and this fluid could be obtained with ICSI pipette from blastocoele (Figure 2) avoiding any cell biopsy of the embryo. This method for blastocyst micropuncture and aspiration of blastocoele fluid has been described previously [38]. Briefly, the expanded day 5 blastocysts were removed from the culture medium and were transferred to a new droplet of blastocyst medium under paraffin oil. The blastocysts were immobilized with a holding pipette and another finely pulled, oil-filled pipette was introduced through the mural trophectoderm to avoid damaging the

inner cell mass. Then the blastocoele fluid was aspirated gently until the blastocyst fully collapsed around the pipette.

The aim of this study was to determine the embryo gender directly from the blastocoele fluid without performing biopsy of embryonic cells. For this purpose the amplification of the multicopy genes *TSPY1* (on the Y chromosome) and *TBCID3* (on chromosome 17) was done. This study opens up the possibility of screening embryos from couples carrying an X-linked disorder to identify male embryos at high risk of disease as well as detect several aneuploidies. However, further studies have to be done in order to validate this approach and to confirm that the accuracy is sufficient for diagnostic purposes [15].

The advantages of performing PGD without embryo biopsy are obvious, but this approach must be considered with caution before any potential clinical application. The group of Cohen [39] has some realistic concerns about this study. These are related to the DNA sample and the doubt that it does not represent the whole embryo since the embryo-free culture media also contain DNA fractions. In addition, it was suggested that this DNA has also been released from abnormal or degenerated cells and therefore could not be as representative as the one released from the intact ones. Moreover, the procedure may be called noninvasive but some damage may occur during the manipulation process and may affect the viability of the blastocyst. Many questions and many doubts arose, but in spite of them, the study of Palini and coauthors is interesting and fascinating and in a provocative manner opens new possibilities for diagnosis of genetic abnormalities in preimplantation embryos by avoiding any cell biopsy during the procedure [39].

5.3. Proteomics, Proteins in Spent Culture Media, and Noninvasive Testing of Embryos.

This failure of embryo implantation from ART is due to both the absence of developmentally competent euploid embryos in an IVF cohort and our inability to select the competent embryo(s). In human, the incorrect number of chromosomes (aneuploidy) is extremely common in human oocytes and increases significantly with advanced maternal age [40, 41]. The embryos generated from these aneuploid gametes have little potential and reduced chance for a viable pregnancy [42]. New recent developments strategies in proteomic technologies and mass spectrometry (MS) have discovered differentially secreted proteins that could lead to noninvasive viability screening, including chromosomal constitution among preimplantation embryos [43]. A recent study of the team of Katz-Jaffe has found a potential biomarker for noninvasive aneuploidy screening called lipocalin-1. The researchers identified this protein in the secretome of human blastocysts in in vitro conditions. An important question could arise: may the protein secretome of human blastocysts be relative to comprehensive chromosome constitution in a noninvasive manner? The method is based on the analysis of proteins of the spent culture media, secreted by a single embryo (secretome). The difference between the euploid and aneuploid blastocyst in a unique secretome signature was evaluated. The preliminary results have been promising and revealed protein differences

that appeared to correlate with chromosome constitution [43]. The protein secretome profiles from individual morphologically similar good-quality blastocysts allowed discrimination between euploid and aneuploid status. In this study, a novel set of nine differentially expressed biomarkers (soluble tumor necrosis factor (TNF), interleukin-10 (IL-10), macrophage-stimulating protein- α (MSP- α), stem cell factor (SCF), chemokine (C-X-C motif) ligand 13 (CXCL13), TNF-related apoptosis inducing ligand receptor 3 (TRAILR3), macrophage inflammatory protein-1 β (MIP-1 β), GM-CSF, and lipocalin-1) was identified with statistical significance and was reproducible in all of the analyzed spent culture media samples [44]. The protein profile of the euploid blastocyst secretome was notably different from the protein profile of the aneuploid blastocyst secretome. These biomarkers characteristically classified chromosome aneuploidy in the cohort of blastocysts available for transfer. The most significant suggestion in this study is that the altered expression levels of lipocalin-1 are related to aneuploidy and not to failed implantation, revealing their potential as a candidate marker for noninvasive aneuploidy screening. The development of this noninvasive technique for determining the euploidy and the competence for development of human embryos by analyzing the spent culture medium could be a powerful tool for embryo selection in ART, but it needs to be researched further.

5.4. Embryo Time-Lapse Monitoring and Aneuploidy. The application of time-lapse imaging of the embryos could be used as a predictor for good implantation and lower aneuploidy rate among the transferable embryos. The widely discussed study of Meseguer et al. [45] reported that morphokinetics of development could be used for prediction of embryo implantation and also could be associated with aneuploidy incidence. The time-lapse observation is an opportunity for optimizing embryo selection based on morphological grading as well as providing novel kinetic parameters, which may further improve accurate selection of viable embryos [46]. A detailed retrospective analysis of time-lapse microscopy results showed that several parameters of developmental dynamics were significantly correlated with subsequent implantation (e.g., time of first and subsequent cleavages as well as the time between cleavages). The most predictive parameters were (1) time of division to 5 cells, t5 (48.8–56.6 h after ICSI); (2) time between division to 3 cells and subsequent division to 4 cells, s2 (≤ 0.76 h); and (3) duration of the second cycle of the cell division, that is, time between division to 2 cells and division to 3 cells, cc2 (≤ 11.9 h).

The embryo aneuploidy, a major cause of IVF failure, has been correlated with specific embryonic morphokinetic variables previously used for the development of an aneuploidy risk classification model. The study of Campbell et al. [47] evaluates the effectiveness and potential impact of this model for unselected IVF patients without embryo biopsy and preimplantation genetic screening (PGS). Embryo outcomes, implantation, fetal heart beat (FHB), and live birth (LB) of 88 transferred blastocysts were compared according to calculated aneuploidy risk classes (low, medium, and high).

A significant difference was seen for FHB ($P < 0.0001$) and LB ($P < 0.01$) rates between embryos classified as low and medium risk. Within the low-risk class, relative increases of 74% and 56%, compared to rates for all blastocysts, were observed for FHB and LB, respectively. This study demonstrated the clinical relevance of the aneuploidy risk classification model and introduced a novel, noninvasive method of embryo selection in order to achieve higher implantation and live birth rates without PGS. By using such unique, noninvasive, and specifically designed embryo selection models, we can now make more informed choices in order to select the most viable embryo to transfer, with the lowest risk of aneuploidy. As a result of this study, the selection of an embryo, classified as low risk, has improved the relative chance of a live birth by 56% over conventional embryo selection.

6. Noninvasive Prenatal Diagnosis and Testing for Pregnant Women

Not only PGD and PGS, but also the noninvasive prenatal diagnosis (NIPD) and noninvasive prenatal testing (NIPT) will offer some new options in prenatal diagnosis for carriers of single gene disorders and chromosomal constitution in fetuses. This will involve fertile patients who reject PGD, patients after PGD for result confirmation, those who reject amniocentesis (AC) or chorionic villus sampling (CVS), patients with previous loss of pregnancy because of the listed procedures, and so forth. These carriers or patients at high risk for chromosomal or monogenic disorder are target groups for the health professionals working in the area of prenatal care.

The cell-free DNA from the fetus has been found in the plasma of pregnant women, and this has been used successfully for noninvasive determination of the fetal gender and fetal RhD genotype in RhD negative women [48–50]. The basis of these tests is the detection of fetal-specific DNA sequences in maternal plasma [51]. The same approach of searching for fetal-specific nucleic acids, such as DNA methylation and mRNA markers in maternal plasma, has been proposed for noninvasive detection of fetal aneuploidies [52, 53] instead of performing invasive sampling of fetal genetic material through the AC or CVS. As source for testing is the circulating in maternal blood 4–6% cell-free fetal DNA/RNA fraction in the 1st trimester of the pregnancy [54].

The noninvasive prenatal diagnosis (NIPD) for single-gene disorders has attracted less interest because it represents a much smaller market opportunity and in the majority of cases has to be provided on disease-specific basis. The methods and workflows are labour-intensive and not easily scalable. Nonetheless, there is a significant need of NIPD of single-gene disorders, and the continuing advances in technology and data analysis should facilitate the expansion of the variety of the disorders where NIPD can be provided. Various methods and platform technologies, as well as technical challenges, were applied to a wider range of genetic disorders. A recent report showed that these tests were mainly

performed for haemophilia [55], beta-thalassaemia [56], and sickle cell anemia [57].

The other test is noninvasive prenatal testing (NIPT), which could be performed before the invasive testing (AC, CVS) for pregnant women who are considered having high risk of trisomy 21. According to Bianchi, this methodology has already been highly applicable for chromosome 21 [58]. There are many findings that besides chromosome 21 and sex chromosome aneuploidies, other chromosomes could also be analyzed (i.e., chromosomes 18 and 13). Nevertheless, the measurements of the proportion of DNA molecules from chromosomes 18 and 13 were far less precise [59]. In the future, further research is required to develop protocols in order to improve the precision for measuring the amount of DNA molecules from chromosomes 18 and 13 [54]. The recent study shows that the routine screening for trisomies: chromosomes 21, 18, and 13 by cell-free DNA (cfDNA) testing at 10 weeks of gestation is feasible and has lower false-positive rates (FPR) than combined testing does, but abnormal results require confirmation by CVS [60]. Time will show if the accuracy of NIPT is as high as the karyotyping after invasive procedure and if the invasive methods can be replaced by noninvasive genetic screening for pregnant women.

Since the ART and reproductive genetics are overlapping fields, necessity for collaboration between the genetic and ART centers has arisen.

7. Minimizing the Genetic Risk for Future ART Generations

Epimutation is also a hot topic, since many PGD laboratories already provide diagnosis for some syndromes and many recent articles search for correlation between ART and some imprinting disorders. Therefore, the field of epigenetic inheritance seems to be a quite interesting area, especially because ART can induce epigenetic variation that might be transmitted to the next generation [61].

The Angelman syndrome is a serious neurodevelopmental disorder [62] although there are no estimates of its absolute risk after ART would be small (1 in 3000). Therefore, it seems unlikely that this would result in many couples requesting ART to decline treatment. Epimutations causing Beckwith-Wiedemann syndrome (BWS) are more frequent than those causing Angelman syndrome but, not in comparison to the risk of serious complications such as exomphalos and embryonal tumours, BWS is usually compatible with normal living.

In order to provide prospective parents with accurate risk information, there is a pressing need to define the absolute risk of imprinting disorders after ART by prospectively following a cohort of ART children. It is acknowledged that many couples will still choose trying for pregnancy despite the known and unknown risks for the child [63].

Suboptimal conditions during oocyte and embryo development may also lead to persistent changes in the epigenome influencing diseases susceptibility later in life. In order to minimize the risk it is clear that the prolongation in vitro culturing to blastocyst stage should be very well optimized. The oocytes with big smooth endoplasmic reticulum (SER)

aggregation might be followed by increased frequency of imprinting disorders. Therefore, their use for fertility treatment must be limited [64]. It is also not known how the embryo biopsy affects the embryo quality. However, apparently the highest risk for rare imprinting disorders in children born following ART remains multiple pregnancy and particularly higher-order multiple pregnancies [65].

Today a successful pregnancy is mainly defined by the outcome at birth; however, the consideration for the consequences of ART conditions for later life remains. The fetuses' adaptations to under nutrition are associated with changes in the concentrations of fetal and placental hormones. Maternal reproductive health is a reflection of events over generations. It is multifactorial, environmentally sensitive and involves genes undergoing reprogramming during the critical period of gametogenesis. It is now widely accepted that the adverse preconceptional and intrauterine environment is associated with epigenetic malprogramming of the fetal metabolism and predisposition to chronic, in particular metabolic disorders, later in life—or the so-called “Barker hypothesis” [66, 67], regardless if the child is born following assisted or natural conception.

There are also some new forthcoming horizons of the meaning of miRNA, siRNA, and piRNA that may play an important role in many biological processes [11], including differentiation of male reproductive cells, and they all may have control over the gene expression and need to be elucidated further.

8. Conclusion

It can be concluded that the preimplantation and prenatal genetic diagnosis and screening are of enormous value for providing healthy baby to couples with genetic disorders or for preventing the repeated spontaneous miscarriages. Nevertheless, there are some concerns about the aggression of the embryo biopsy by itself and potential epigenetic disturbance; therefore, there are some new noninvasive approaches for evaluation of the genetic status of human embryos and fetuses by a nondirect manner. Some of these approaches are interesting and seem to be quite promising, but further research is needed to elucidate if some of them could replace the existing procedures in the future or can only have additive value in diagnosis.

Conflict of Interests

The author declares that there is no financial or any conflict of interests related to this paper.

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Clinical Study

Comparative Genomic Hybridization Selection of Blastocysts for Repeated Implantation Failure Treatment: A Pilot Study

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The aim of this study is to determine if the use of preimplantation genetic screening (PGS) by array comparative genomic hybridization (array CGH) and transfer of a single euploid blastocyst in patients with repeated implantation failure (RIF) can improve clinical results. Three patient groups are compared: 43 couples with RIF for whom embryos were selected by array CGH (group RIF-PGS), 33 couples with the same history for whom array CGH was not performed (group RIF NO PGS), and 45 good prognosis infertile couples with array CGH selected embryos (group NO RIF PGS). A single euploid blastocyst was transferred in groups RIF-PGS and NO RIF PGS. Array CGH was not performed in group RIF NO PGS in which 1-2 blastocysts were transferred. One monoembryonic sac with heartbeat was found in 28 patients of group RIF PGS and 31 patients of group NO RIF PGS showing similar clinical pregnancy and implantation rates (68.3% and 70.5%, resp.). In contrast, an embryonic sac with heartbeat was only detected in 7 (21.2%) patients of group RIF NO PGS. In conclusion, PGS by array CGH with single euploid blastocyst transfer appears to be a successful strategy for patients with multiple failed IVF attempts.

1. Introduction

According to ESHRE PGD consortium, repeated implantation failure (RIF) is defined as the absence of a gestational sac on ultrasound at 5 or more weeks after embryo transfer (ET) after 3 embryo transfers with high quality embryos or after the transfer of ≥ 10 embryos in multiple transfers [1]. Repeated implantation failure can be caused by both maternal and embryonic factors [2]. Intrauterine pathologic conditions, such as polyps, intrauterine adhesions, submucous myomas, and a septated or subseptated uterus, have been demonstrated to disturb embryo implantation [3]. Endometrial receptivity is also decreased with reduced endometrial thickness and/or altered expression of endometrial adhesive molecules [3]. Hydrosalpinx, autoimmune conditions, thrombophilia, inadequate ET methods, or altered life styles are all recognized potential causes of RIF [4]. Sperm DNA damage,

zona pellucida hardening, inadequate culture conditions, and suboptimal embryo development can also play a significant role in the etiology of RIF [5]. Development of aneuploid embryos, independently of their morphological quality, is another well-recognized cause of RIF in IVF [6].

Several studies have demonstrated that at least 15% of patients with high-order RIF have an increased frequency of female partner chromosomal abnormalities [7]. Aneuploidy can arise during meiosis or after fertilization. Most meiotic errors are derived from oocytes and the frequency of oocyte aneuploidy increases with advancing female age [8]. Chromosomal abnormalities arising at cleavage stages mostly occur during the first three mitotic divisions, leading to chromosomal mosaicism [9–11]. For these reasons, it has been suggested that the use of preimplantation genetic screening (PGS) to select and transfer chromosomally normal embryos

may lead to improved IVF results in this group of patients [12].

Most available data on the impact of PGS on the outcome of IVF in cases of RIF have been obtained with the use of fluorescent *in situ* hybridization (FISH) to assess up to 12 chromosomes in single embryonic blastomeres [13] and are ambiguous and inconclusive. Embryo implantation and live birth rates are not increased after transfer of embryos screened by FISH-based PGS, probably because the limited number of analyzed chromosomes is not sufficient [14, 15]. In fact, it has been demonstrated that aneuploidies may occur in preimplantation embryos in any of the 23 chromosomes indicating that aneuploidy screening of all chromosomes is necessary to determine whether an embryo is chromosomally normal [16–19]. Genome-wide approaches are certainly more comprehensive than FISH (23 compared with ≤ 12 chromosomes, resp.) and some microarray based methods have shown significantly improved consistency [17–21] and predictive value for aneuploidy diagnosis [22, 23].

The possibility to examine simultaneously all the chromosomes by array comparative genomic hybridization (array CGH) in a few cells or in a single cell [24] provided a new opportunity to evaluate the embryos by PGS in patients with RIF. Array CGH techniques have been recently introduced into current routine PGS laboratory practices. This technique has been adapted for comprehensive molecular cytogenetic analysis of metaphase II oocytes and their polar bodies [25], cleavage stage embryos [17, 26], and blastocysts [27]. In this latter case, biopsy of trophoctoderm (TE) cells can have several advantages with respect to biopsy of blastomeres at day 3 of embryo development [28]. Because a larger number of cells can be biopsied from a blastocyst, it is expected that more accurate information can be obtained as compared to one-cell or a few-cell biopsy from cleavage stage embryos, thus reducing the risk of misdiagnosis of embryonic chromosomal mosaicism. Moreover, the TE cell biopsy does not produce any mechanical or functional damage relevant to further development of the biopsied blastocyst [6]. Some scientific studies demonstrate that the cells sampled during TE biopsy are representative of the embryonic inner cell mass (ICM) [27]. Depending on the speed of blastocyst development, the biopsy can be done on day 5, 6, or 7. Only in the first case the transfer of the euploid blastocyst can be performed in the fresh cycle. When the biopsies are carried out on day 6 or 7, it is necessary to cryopreserve the blastocysts by vitrification and delay of the transfer for a subsequent cycle. Initial studies on the clinical use of array CGH in embryos have documented improved identification of abnormalities as well as high pregnancy outcomes following transfer of screened embryos [17, 27, 29, 30]. In particular, a recent study [29], evaluating the efficacy of single embryo transfer (SET) coupled with comprehensive chromosome screening (CCS) in an infertile population, indicated that 23 chromosomes PGS increases ongoing pregnancy rate and reduces the miscarriage rate, compared with traditional blastocyst SET.

In spite of the encouraging data with PGS on cells obtained by TE biopsy, the clinical results of this intervention in a large group of patients with RIF have not yet been clearly demonstrated. Moreover, in the previously published

studies more than one euploid blastocyst was transferred and, consequently, many twin gestations were obtained [6, 30]. Additionally one of the most important efforts of IVF practitioners is to reduce the risk of multiple pregnancies maintaining acceptable overall live birth rates following IVF-ET. Elective single embryo transfer is the adopted strategy to reach this scope [31]. Recent studies have demonstrated that single euploid blastocyst transfer gives better clinical results than single morphologically selected blastocyst transfer, both in fresh and frozen-thawed cycles in good prognosis patients [32, 33].

The aim of this study is to assess the clinical pregnancy and implantation rates after transfer of a single euploid blastocyst in a group of patients of <36 years of age and without a history of recurrent miscarriages (RM). The results obtained in this group are compared with a similar group of RIF patients in whom PGS was not performed (negative control) and with a group of good prognosis patients after PGS (positive control).

2. Materials and Methods

2.1. Inclusion Criteria, Informed Consent, and Ethical Considerations. This study was performed between March, 2012, and March, 2013. A total of 121 couples were involved, including 76 couples with a history of 3–9 (mean 4.9) implantation failures in previous IVF attempts (RIF group) and 45 couples undergoing their first IVF attempt without any detected problem of ovarian reserve and uterine receptivity or sperm quantity and quality (good prognosis group). The couples of the RIF group were further divided into two subgroups: those couples who consented to have their embryos analyzed by array CGH with subsequent transfer of a single euploid blastocyst formed group RIF PGS, whereas group RIF NO PGS (negative control) consisted of couples in whom embryos were not analyzed by array CGH and in whom all available 1–2 blastocysts with the best morphology were transferred. The good prognosis couples who chose array CHG to be performed with their embryos followed by single blastocyst transfer formed group NO RIF PGS (positive control).

All female patients were less than 36 years old. Their ovarian reserve was evaluated before starting ovarian stimulation by determining antral follicle count (AFC), by transvaginal ultrasound on the first days of the cycle (2–5) and by day 3 FSH and anti-Müllerian hormone (AMH) dosage [34]. Patients with abnormal karyotype, uterine abnormalities, autoimmune conditions, thrombophilia, severe endometriosis, and reduced ovarian reserve were excluded from the study.

Seminal fluid examination of male partners was performed after 3–5 days of sexual abstinence according to the World Health Organization (WHO) recommendations [35]. All male patients with severe infertility (<500,000 motile sperm/mL after preparation) or with high sperm DNA fragmentation were excluded [36].

All the three groups were well matched for all relevant male and female clinical parameters (Table 1). A written informed consent was obtained from each couple after

TABLE 1: Clinical parameters analyzed for the three groups (SD, standard deviation).

	Group RIF PGS (n = 43)	Group RIF NO PGS (n = 33)	Group NO RIF PGS (n = 45)	P
Mean female age \pm SD	32.8 \pm 3.1	31.5 \pm 2.9	31.7 \pm 2.9	NS
Mean FSH \pm SD (day 3, nv 3–10 mUI/mL)	7.8 \pm 1.7	7.7 \pm 0.7	8.1 \pm 1.8	NS
Mean AMH \pm SD (nv 0, 2–5, 5 ng/mL)	4.1 \pm 1.1	5.2 \pm 2.4	4.6 \pm 1.2	NS
Mean number of antral follicles \pm SD	12.4 \pm 1.9	12.9 \pm 2.8	13.8 \pm 2.1	NS
Mean sperm count (M/mL) \pm SD	9.8 \pm 2.1	14.4 \pm 9.2	10.1 \pm 2.0	NS
Mean sperm motility (%) \pm SD	50.7 \pm 16.8	47.4 \pm 15.5	51.2 \pm 16.9	NS
Mean sperm morphology (%) \pm SD	5.5 \pm 2.3	6.7 \pm 2.9	5.1 \pm 2.1	NS

counseling about array CGH. The study was approved by the Institutional Review Board of the European Hospital Clinic and GENOMA Laboratory. All experimentations were performed according to the Helsinki Declaration of 1975 and its modifications.

2.2. IVF Clinical and Laboratory Protocols. Controlled ovarian stimulation was performed using recombinant FSH (Gonal F, Merck Serono, Geneva, Switzerland) and a long gonadotropin-releasing hormone (GnRH) agonist suppression protocol or GnRH antagonist flexible protocol according to ovarian reserve and AMH values as described elsewhere [37–39]. Recombinant FSH starting dose was calculated taking into account the patient's age, body mass index, AFC, and AMH values of the patients. Periodic transvaginal ultrasound scans were performed to assess the number and the mean diameter of the growing follicles. Together with serum estradiol levels, these data were used to adjust the recombinant FSH dose. When at least 3 follicles reached 19 mm in diameter, hCG (Gonasi, 10,000 IU, IBSA, Lodi, Italy) was administered by intramuscular injection. Oocytes were retrieved 36–38 h later by ultrasound-guided transvaginal follicular puncture.

After retrieval oocytes were incubated for 2–3 h at 37°C under the gas phase of 5% O₂ and 6% CO₂ before starting the removal of the surrounding cumulus oophorus and corona radiata cells [40, 41]. The oocyte denudation was performed by a brief exposure to 40 IU/mL hyaluronidase solution in fertilization medium (Sage In-Vitro Fertilization, Inc., Trumbull, CT, USA), followed by mechanical removal of all the remaining cumulus and corona cells with the use of plastic pipettes of defined diameters (denuding pipette; COOK Ireland Ltd., Limerick, Ireland). The denudation procedure was completed between 38 and 40 hours after hCG administration, and the oocytes were treated by ICSI immediately thereafter. Particular attention was paid to the removal of all adhering cumulus and coronal cells with the aim to avoid maternal DNA contamination during the amplification steps.

ICSI was performed 38–40 hours after hCG administration, using previously described techniques and instrumentation [42]. Fertilization was considered normal when two

clearly distinct pronuclei and two polar bodies were present on day 1, 16–18 h after ICSI as described elsewhere [43]. Embryo culture was carried out in cleavage medium under mineral oil (Sage In-Vitro Fertilization, Inc., Trumbull, CT, USA) up to day 3 of embryo development followed by blastocyst medium (Sage In-Vitro Fertilization, Inc., Trumbull, CT, USA) up to day 5, 6, or 7 at 37°C and under 5% O₂ and 6% CO₂. Embryo culture was performed in Embryoscope or in a mini-incubator (SANYO), where all embryos from each patient were kept separately from other couples throughout the culture duration.

2.3. Biopsy Procedure and Cells Preparation. On day 3, when the embryos reached the 6–8 cell stage, a noncontact 1.48 μ m diode laser [27] was used to create a circular 6–9 μ m diameter opening in the zona pellucida in order to allow the biopsy of 5–10 herniated TE cells on day 5 or 6, depending on the speed of blastocyst development. On the day of biopsy, TE cells were gently aspirated into the biopsy pipette (biopsy pipette; COOK Ireland Ltd., Limerick, Ireland) followed, if necessary, by a laser assisted removal from the body of the blastocyst. The obtained TE cells were washed in sterile phosphate-buffered saline solution (PBS) and then placed in microcentrifuge tubes containing 2 μ L of PBS, spinned down for few seconds and sent to GENOMA Laboratory for analysis.

2.4. Array CGH Protocol. TE cells were lysed, and genomic DNA was amplified using the SurePlex DNA Amplification System (BlueGnome, Cambridge, UK), according to the manufacturer's instructions. Whole Genome Amplification (WGA) products were processed as reported elsewhere [17] according to the BlueGnome 24sure V3 protocol (available at <http://www.cytochip.com/>). Briefly, WGA products were fluorescently labelled and competitively hybridized to 24sure V3 arrays (BlueGnome, Cambridge, UK) with a matched control in an array CGH experiment format. A laser scanner InnoScanw 710 AL (INNOPSYS, Carbonne, France) was used to excite the hybridized fluorophores and read and store the resulting images of the hybridization. Scanned images were then analysed and quantified by algorithm fixed settings in

BlueFuse Multi Software (BlueGnome, Cambridge, UK), a software package that performed the steps of grid placement, quantification, normalization, and postprocessing automatically. The whole procedure was completed within 12–24 h, and the results were obtained in time for an embryo transfer at day 6, in a fresh cycle.

2.5. Embryo and Blastocyst Grading. Embryo morphology was checked on days 2 and 3 using the scoring system reported elsewhere [40]. Briefly, for each embryo, the number and size of the blastomeres were observed, as well as the percentage of anucleated fragments. Cleaved embryos with no more than 20% of their volume occupied by fragments and with equal-sized blastomeres were considered type A. When the percentage of the volume filled with fragments was between 20% and 50%, the embryos were considered type B. Finally, when >50% fragments were present, embryos were considered type C.

Embryos reaching the blastocyst stage were graded by using the system of Gardner and Schoolcraft [44]. Blastocysts were given a number based on the degree of expansion and hatching status (from 1 to 6): (1) early blastocyst: the blastocoel accounts for less than one-half of the volume of the embryo; (2) blastocyst: the blastocoel occupies more than one-half of the volume of the embryo; (3) full blastocyst: the blastocoel fills the embryo completely; (4) expanded blastocyst: the blastocoel is now larger than the early embryo, and the zona pellucida has begun to thin; (5) hatching blastocyst: TE cells have begun to herniate through the zona pellucida; and (6) hatched blastocyst: the blastocyst has completely escaped from the zona pellucida.

For fully developed blastocysts (grades 3–6), a second scoring step was performed under an inverted microscope to assess the inner cell mass and the trophectoderm. For the ICM, the following descriptions are used: (a) tightly packed with many cells, (b) loosely grouped with several cells, and (c) very few cells. For the TE, the following grading is used: (a) many cells forming a cohesive epithelium, (b) few cells forming a loose epithelium, and (c) very few large cells.

2.6. Blastocyst Vitrification and Warming. Vitrification procedure was used to cryopreserve all surplus blastocysts, both euploid ones, for later use, and aneuploid ones whose storage is dictated by the Italian law which forbids any embryo destruction. All embryos that reached the blastocyst stage on day 6 or 7 were also vitrified. Vitrification was carried out with the use of the Kuwayama protocol with Cryotop as support as previously described [45]. In brief, blastocysts were placed in equilibration solution (Kitazato Vitrification Kit, BioPharma, Shizuoka, Japan) containing 7.5% ethylene glycol and 7.5% dimethyl sulfoxide for 15 minutes at room temperature and then moved to a vitrification solution composed of 15% ethylene glycol, 15% dimethyl sulfoxide, and 0.5 mol/L sucrose for 30 to 60 seconds. Individual blastocysts were loaded onto the polypropylene strip of the Cryotop in a volume of <0.1 μ L, quickly plunged into liquid nitrogen, capped with a protective cover, and stored in a liquid N₂ storage tank at –196°C.

Warming was carried out with the use of the Kuwayama protocol as previously described [45]. In brief, warming was performed by placing the Cryotop in a thawing solution (Kitazato Warming Kit, BioPharma, Shizuoka, Japan) of 1 mol/L sucrose for 45 to 60 seconds at 37°C. Blastocysts then were transferred to a dilution solution of 0.5 mol/L sucrose for 3 minutes, followed by washing with medium without sucrose for 5 minutes. The surviving blastocysts were incubated for 4 hours before their transfer to the uterus. Endometrial preparation was carried out as described previously [46].

2.7. Blastocyst Transfer, Luteal Phase Support, and Pregnancy Determination. Single frozen-thawed embryo transfer (FET) was performed in patients prepared by combining gonadotropin-releasing hormone agonist and estrogen pills (Progynova, Bayer, New Zealand Limited, Auckland) or in a spontaneous cycle. Fresh single embryo transfer (SET) was performed in the morning of day 6 after ICSI. All transfer procedures were carried out with the use of a catheter (Wallace, Smits-Medical, Dublin, Ireland) under direct ultrasound guidance as previously described [47]. In groups RIF PGS and NO RIF PGS only euploid blastocysts were selected for transfer. If several euploid blastocysts were available in these two groups, the best-morphology one was selected for transfer. In group RIF NO PGS (without ploidy evaluation), 1–2 best-morphology blastocysts were transferred. No blastocyst transfer was done with endometrium thickness of <7 mm.

Intramuscular administration of progesterone in oil (Prontogest, IBSA, Lodi, Italy) was initiated 6–7 days before embryo transfer and continued until the first serum β -hCG determination. Biochemical pregnancy was confirmed by the detection of increasing β -hCG levels 9 days after blastocyst transfer. Clinical pregnancies were defined as those showing the presence of an intrauterine gestational sac determined by transvaginal ultrasound examination at 7 weeks of gestation. Implantation rate was defined as the number of gestational sacs per transferred embryos [48].

2.8. Statistical Methods. Required sample size was estimated to evaluate the implantation rate after the transfer of a single euploid blastocyst in patients with only repeated implantation failure without advanced maternal age or previous miscarriages. Considering a prevalence of 50% of implantation rate, with an error of 15% and 95% confidence interval, 43 couples are needed. The same number of good prognosis couples is considered as positive control group (C). Only 33 couples were available for the negative control group (B), but the number of embryos transferred in this group was similar to that in groups A and C.

In order to compare the maternal age, FSH, AMH, number of antral follicles, sperm count, sperm motility, and sperm morphology between the three groups ANOVA was used. Chi-square test, or Fisher's exact test when necessary, was used to compare categorical data. Bonferroni correction was used in post hoc analysis comparing only groups A versus B and groups A versus C. Exact confidence interval at 95% (95% CI) was reported regarding the principal outcome. A *P* value <0.05 was considered statistically significant. Stata 12.1 was used for all analysis.

TABLE 2: Oocytes, embryos, and blastocysts obtained in the three groups.

	Group RIF PGS (<i>n</i> = 43)	Group RIF NO PGS (<i>n</i> = 33)	Group NO RIF PGS (<i>n</i> = 45)	<i>P</i>
Number of oocytes	645	519	681	
Metaphase II	530 (82.2%)	454 (87.5%)	556 (81.6%)	0.014 A versus B 0.026* A versus C 1.00*
Fertilized oocytes	433 (81.7%)	373 (82.2%)	451 (81.1%)	0.912
Embryos	392 (90.5%)	337 (90.3%)	408 (90.5%)	0.996
Day 3 grade A	236 (60.2%)	210 (62.3%)	246 (60.3%)	0.013
Day 3 grade B	105 (26.8%)	64 (19.0%)	112 (27.5%)	A versus B 0.026*
Day 3 grade C	51 (13.0%)	63 (18.7%)	50 (12.3%)	A versus C 1.00*
Blastocysts	190 (48.5%)	171 (50.7%)	257 (63.0%)	<0.001 A versus B 1.00* A versus C 0.001*
Day 5	83 (43.7%)	87 (50.9%)	108 (42.0%)	<0.001
Day 6	96 (50.5%)	51 (29.8%)	125 (48.6%)	A versus B 0.001*
Day 7	11 (5.8%)	33 (19.3%)	24 (9.3%)	A versus C 0.770*

* After Bonferroni's correction.

3. Results

All clinical parameters, including age, day 3 serum FSH concentration, serum AMH concentration and days 2–5 AFC for the female partners, and sperm count, motility, and morphology for the male partner, were similar in groups A (*n* = 43), B (*n* = 33), and C (*n* = 45) (Table 1).

In group RIF PGS, 645 oocytes were collected; 530 of them were in metaphase II stage (82.2%) and all of them were injected and 433 fertilized normally (81.7%) resulting in 392 embryos (90.5). In group RIF NO PGS, 519 oocytes were collected; 454 of them were metaphase II (87.5%) and all of them were injected and 373 fertilized normally (82.2%) resulting in 337 embryos (90.3%). In group NO RIF PGS, 681 oocytes were collected; 556 of them were metaphase II (81.6%) and all of them were injected and 451 fertilized normally (81.1%) resulting in 408 embryos (90.5%) (Table 2).

In group RIF PGS, day 3 embryo quality was A = 236 (60.2%), B = 105 (26.8%), and C = 51 (13.0%). A total of 190 blastocysts were obtained (48.5%): 83 blastocysts were obtained on day 5 (43.7%), 96 on day 6 (50.5%), and 11 on day 7 (5.8%). In group RIF NO PGS day 3 embryo quality was A = 210 (62.3%), B = 64 (19.0%), and C = 63 (18.7%). A total of 171 blastocysts were obtained (50.7%): 87 blastocysts were obtained on day 5 (50.9%), 51 on day 6 (29.8%), and 33 on day 7 (19.3%). In group NO RIF PGS, day 3 embryo quality was A = 246 (60.3%), B = 112 (27.5%), and C = 50 (12.3%). A total of 257 blastocysts were obtained (63.0%): 108 blastocysts were obtained on day 5 (42.0%), 125 on day 6 (48.6%), and 24 on day 7 (9.3%) (Table 2).

In groups RIF PGS and NO RIF PGS, a total of 447 blastocysts were obtained on days 5–7 (Table 2). All blastocysts were biopsied in both of these groups. The percentage of embryos reaching the blastocyst stage in group RIF PGS was lower as compared to group NO RIF PGS,

and no statistical difference was observed in the other above-mentioned biological parameters between the two groups (*P* > 0.05) (Table 2). In group RIF NO PGS the total number of blastocysts (days 5–7) was 171. Similar to group RIF PGS, the percentage of blastocysts in group RIF NO PGS was lower than that in group NO RIF PGS (Table 2). No blastocyst was biopsied in this group.

In group RIF PGS, array CGH yielded interpretable results for 182 out of the 190 blastocysts, leading to a diagnostic efficiency of 95.8%. In group NO RIF PGS, array CGH yielded interpretable results for 245 out of the 257 blastocysts, with a diagnostic efficiency of 95.3% (Table 3).

In group RIF PGS, 84 blastocysts were classified as euploid (46.2%), whereas 98 were classified as aneuploid, resulting in an abnormality rate of 53.8%. Of the aneuploid blastocysts, 35.7% (*N* = 35) were complex aneuploidies, 22.4% (*N* = 22) carried two chromosome errors, 19.4% (*N* = 19) trisomies, 21.4% (*N* = 21) monosomies, and 1.0% (*N* = 1) mosaicisms (Table 3). In group NO RIF PGS, 127 blastocysts were classified as euploid (51.8%), whereas 118 were classified as aneuploid, resulting in an abnormality rate of 48.2%. Of the aneuploid blastocysts, 27.1% (*N* = 32) were complex aneuploidies, 25.4% (*N* = 30) carried two chromosome errors, 21.2% (*N* = 25) trisomies, 22.9% (*N* = 27) monosomies, and 3.4% (*N* = 4) mosaicisms. No statistical differences were observed in aneuploidy rates and types of aneuploidies between the two groups (*P* = 0.245; *P* = 0.173) (Table 3).

Forty-one couples received a single euploid blastocyst replacement in group RIF PGS and 44 in group NO RIF PGS. In group RIF PGS, 15 patients received a single fresh blastocyst transfer (36.6%) and 26 patients received a single frozen-thawed blastocyst transfer (63.4%), 14 in natural cycle and 12 after endometrial preparation with exogenous estrogen (Table 4). In group NO RIF PGS, 19 patients received a single fresh blastocyst transfer (43.2%) and 25 patients

TABLE 3: Array comparative genomic hybridization results.

	Group RIF PGS (n = 43)	Group RIF NO PGS (n = 33)	Group NO RIF PGS (n = 45)	P
Blastocysts with amplification (efficiency %)	182/190 (95.8%)	Not applicable	245/257 (95.3%)	0.817
Euploidy	84/182 (46.2%)	Not determined	127/245 (51.8%)	0.245
Aneuploidy	98/182 (53.8%)	Not determined	118/245 (48.2%)	
Complex aneuploidy	35 (35.7%)	Not determined	32 (27.1%)	
Two chromosome errors	22 (22.4%)	Not determined	30 (25.4%)	
Trisomic	19 (19.4%)	Not determined	25 (21.2%)	
Monosomic	21 (21.4%)	Not determined	27 (22.9%)	
Mosaicism	1 (1.0%)	Not determined	4 (3.4%)	0.610

TABLE 4: Clinical results.

	Group RIF PGS (n = 43)	Group RIF NO PGS (n = 33)	Group NO RIF PGS (n = 45)	P
Total single embryo transfer	41	25	44	<0.001 A versus B 0.001* A versus C 1.00*
Total double embryo transfer	0	8	0	
Total fresh embryo transfer	15 (36.6%)	33 (100%)	19 (43.2%)	
Total frozen embryo transfer	26 (63.4%)	0	25 (56.8%)	<0.001 A versus B 0.001* A versus C 1.00*
Frozen embryo transfer in natural cycle	14 (53.8%)		12 (48.0%)	
Frozen embryo transfer with endometrial preparation	12 (46.2%)		13 (52.0%)	
Total embryos transferred	41	41	44	<0.001 A versus B 0.001* A versus C 1.00*
bHCG positive	34 (82.9%)	9 (27.3%)	37 (84.1%)	
Implantation (IR)	28 (68.3%)	9 (22.0)	31 (70.5%)	
Clinical pregnancy (CPR)	28 (68.3%)	7 (21.2%)	31 (70.5%)	0.609
Biochemical pregnancy	2 (4.9%)	2 (6.1%)	4 (9.1%)	
Anembryonic pregnancy	3 (7.3%)	0	2 (4.5%)	
Tubal pregnancy	1 (2.4%)	0	0	
Spontaneous abortion	0	0	0	

* After Bonferroni's correction.

received a single frozen-thawed blastocyst transfer (56.8%), 12 in natural cycle and 13 after endometrial preparation (Table 4). All the cryopreserved blastocysts survived after thawing in both groups (100%).

In group RIF NO PGS, 41 blastocysts were transferred and all of them in the fresh state. There were 25 single embryo transfers and 8 double embryo transfers (Table 4).

β -hCG test was positive for 34 couples in group RIF PGS (82.9%) and for 37 in group NO RIF PGS (84.1%) ($P = 0.331$). In contrast, only 9 couples (27.3%) had a positive β -hCG test in group RIF NO PGS, significantly less as compared with both group RIF PGS and group NO RIF PGS ($P < 0.001$) (Table 4).

Ultrasound examination, which took place 7 weeks after ET, revealed one monoembryonic sac with heartbeat in 28 patients of group RIF PGS, in 7 patients of group RIF NO PGS, and in 31 patients of group NO RIF PGS. Clinical pregnancy and implantation rates, respectively, were 68.3% and 68.3% in group RIF PGS, 22.0% and 21.2% in group RIF NO PGS, and 70.5% and 70.5% in group NO RIF PGS (Figure 1). In group RIF PGS, 2 pregnancies were biochemical, 3 anembryonic, and one tubal. In group RIF NO PGS, 2 pregnancies were biochemical, none anembryonic, and none tubal. In group NO RIF PGS, 4 pregnancies were biochemical, 2 anembryonic, and none tubal. There were no spontaneous abortions in any group (Table 4).

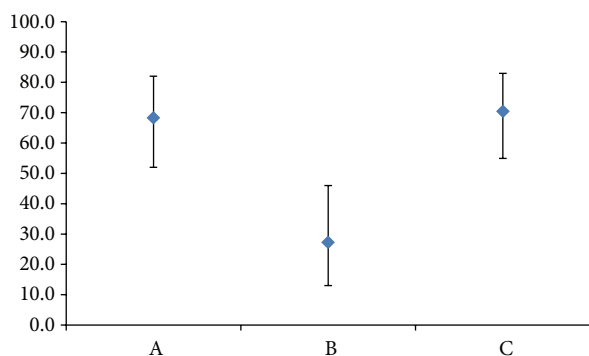


FIGURE 1: Implantation rates in groups RIF PGS (A), RIF NO PGS (B), and NO RIF PGS (C).

There were no statistically significant differences in the clinical pregnancy and implantation rates between fresh and frozen cycles for any group (Table 4).

In contrast only 7 clinical pregnancies were achieved in group RIF NO PGS, all of them monoembryonic. Clinical pregnancy rate (21.2%) and implantation rate (22.0%) in group RIF NO PGS were significantly lower ($P < 0.001$) as compared to both group RIF PGS and group NO RIF PGS.

All patients with clinical pregnancy in any group have delivered a healthy child.

4. Discussion

Repeated implantation failure (RIF) refers to a situation when good quality embryos fail to implant in several subsequent IVF treatment cycles. The mechanism of embryo implantation failure is poorly understood, but it is clear that it can involve both maternal and embryonic factors.

Failure of implantation due to embryonic causes is associated with either genetic abnormalities or other factors intrinsic to the embryo that impair its ability to develop in the uterus, to hatch, and to implant. A high incidence of complex chromosome abnormalities has been discovered in cleaving embryos from patients with RIF [49, 50].

This is the first study to show that embryo selection by array CGH, performed at the blastocyst stage, has the potential to improve the implantation rate and the success rate of IVF cycles in patients with RIF. In fact, the group of couples with single euploid blastocyst transfer compares favourably with the clinically comparable group of couples in whom the transferred blastocysts were selected merely on the base of morphology (negative control), even though more blastocysts were transferred in the latter group. These results are not unexpected since it has been shown that embryo development to blastocyst stage does not represent by itself an absolute selective barrier against chromosome errors [51] even though the rate of aneuploidy is significantly lower for blastocysts than for embryos at earlier stages (38.8% versus 51%) [52].

Moreover, the success rate of IVF after single euploid blastocyst transfer gives similar results for couples with RIF

and for good prognosis couples in their first IVF attempt (positive control). This latter comparison demonstrates, for the first time, that embryo aneuploidies are by far the main cause of RIF as compared with other possible etiologies. The present data also confirm that trophectoderm biopsy does not impair the implantation potential, in agreement with a previous study [53]. The identification of the most viable embryos within a cohort is one of the main goals in IVF in order to perform a single embryo transfer and avoid multiple pregnancies. A recent study reports that the cohort size is not significantly associated with the aneuploidy rate [54]. Several morphological scoring systems have been designed to select the most viable embryos in a cohort, by analyzing pronuclear-stage zygotes [43], cleavage-stage embryos [42], and blastocysts [44]. Our study, in agreement with others, shows that morphological criteria of embryo selection are not fully representative of the genetic health of the embryo at the blastocyst stage [55, 56]. For this reason, it appears to be difficult to further improve clinical pregnancy rates with purely morphological criteria, even though blastomere fragmentation and multinucleation in early cleaving embryos have been shown to be associated with an increased risk of anomalies in chromosome segregation, leading to chromosomal aberrations [57, 58]. This is even more evident in poor prognosis patients such as those with advanced maternal age (AMA), with recurrent abortion (RA), and, as in the present study, with RIF.

In this study, all the embryos were cultured until the blastocyst stage. The number of blastocysts available on days 5, 6, and 7 was similar for groups RIF PGS and NO RIF PGS. On the other hand, less blastocysts developed to day 6 and more blastocysts developed to day 7 in group RIF NO PGS. These differences may be due to the fact that embryos of group NO RIF PGS were not biopsied. However, the mechanism of these differences is not clear for the time being. It has been demonstrated that only embryos with highest implantation potential are able to achieve this stage and that embryos affected by chaotic mosaicism have a reduced capacity for forming a blastocyst [59, 60]. This can explain the reduced blastocysts development in RIF patients with respect to good prognosis couples obtained in the present study. On the other hand, many studies have confirmed that chromosome abnormalities and mosaicism remain common at the blastocyst stage, although aneuploidy rates are reduced compared with the cleavage stages [11]. There has been an increasing interest in defining the types of chromosome errors compatible with blastocyst development. The results of this study confirm that even embryos with the most severe chromosomal abnormalities are often capable of developing up to the blastocyst stage [10], although the aneuploidy rate in blastocysts might be lower than that obtained, using similar methods, in early cleaving embryos [61]. These observations confirm and extend previously published data on blastocyst biopsy and array CGH testing [30, 62].

The accuracy and efficiency of the identification of blastocyst chromosome abnormalities by TE cell biopsy have been validated by different studies showing that TE samples provide accurate information of the chromosome constitution

of the inner cell mass in the vast majority of cases [63]. The risk of misdiagnosis of chromosomal mosaicism is reduced by the analysis of several cells obtained by TE biopsy (5–10), and it has been demonstrated that most mosaic blastocysts do not contain any normal cells [62]. Clinical data suggest that an approach combining blastocyst biopsy and comprehensive chromosome screening using array CGH or microarray CGH may represent the optimal approach for preimplantation genetic screening [15, 52].

In the investigation of chromosome abnormalities at the blastocyst stage, the presence of aneuploidy for three or more chromosomes has been defined as complex chromosome abnormality [6]. Since complex chromosome abnormalities have a relatively low incidence in oocytes, their presence in cleaving embryos and blastocysts is likely to be of postzygotic origin, resulting from abnormal mitotic divisions of embryonic cells [6]. The factors responsible for these abnormalities are not known, and it has been suggested that the lack or dysfunction of cell cycle checkpoints at different cleavage stages of embryo development may be implicated [11]. Complex chromosome abnormalities can be identified more accurately with the use of array CGH as compared with FISH because array CGH is capable of providing data about the whole genome, whereas the capacity of FISH is relatively limited. There appears to be no predilection of any individual chromosome to be involved in a complex chromosome abnormality. Consequently, the limitation of evaluating one, two, or a few chromosomes, as is the case in most FISH protocols, bears the risk of missing an important complex chromosome abnormality or of misinterpreting it as a simple aneuploidy. This may explain the contradictory results obtained by FISH in RIF patients [6].

Our study was intended to be a preliminary and rapid clinical evaluation for a new treatment option in RIF patients with a high number of previous failed attempts in order to obtain successful clinical results avoiding the potential risk of multiple ovarian stimulations. To the best of our knowledge, this is also the largest genetic study on embryos from RIF patients without advanced maternal age or multiple abortions.

Unlike previous studies [64, 65], our data do not highlight any detrimental effect of day 6 transfer on the blastocyst implantation rate. Larger and RCT studies are needed to confirm these preliminary observations.

In conclusion, the results of this study show that array CGH has the potential to provide high rates of embryo implantation after transfer of a single euploid blastocyst in patients with a history of RIF following transfer of good-morphology embryos.

Thus, the combined use of array CGH and single blastocyst transfer can provide an efficient tool for improving IVF clinical outcomes in RIF patients without increasing the number of transferred embryos and the risk of unwanted multiple pregnancies. In a wider perspective, this technique can also be used in patients who, independently of a RIF history, wish to limit the number of transferred embryos to a single one for different personal, social, or economic reasons.

Authors' Contribution

Ermanno Greco played a central role in patient management and was involved in study conception and design, data interpretation, drafting the paper, and final approval. Sara Bono played a central role in all genetic procedures. Alessandra Ruberti played a central role in all laboratory procedures such as ICSI and blastocyst biopsy and transfer. Anna Maria Lobascio played a central role in all laboratory procedures such as ICSI and blastocyst biopsy and transfer, freezing, and thawing procedures. Pierfrancesco Greco was involved in data interpretation and final approval. Anil Biricik was involved in genetic analysis and interpretation of data. Letizia Spizzichino played a central role in all genetic procedures. Alessia Greco played a central role in psychological counseling of the patients enrolled in this study. Jan Tesarik was involved in clinical and laboratory procedures for a part of the patients, in data interpretation and critical revision of the paper. Maria Giulia Minasi played a role in laboratory procedures and was involved in the acquisition of data, data analyses, the critical revision of the paper, and final approval. Francesco Fiorentino was involved in genetic analysis of results, the critical revision of the paper, and final approval.

Conflict of Interests

No conflict of interests has to be declared by any of the authors regarding the material discussed in the paper.

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Review Article

Cryopreservation of Embryos and Oocytes in Human Assisted Reproduction

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Both sperm and embryo cryopreservation have become routine procedures in human assisted reproduction and oocyte cryopreservation is being introduced into clinical practice and is getting more and more widely used. Embryo cryopreservation has decreased the number of fresh embryo transfers and maximized the effectiveness of the IVF cycle. The data shows that women who had transfers of fresh and frozen embryos obtained 8% additional births by using their cryopreserved embryos. Oocyte cryopreservation offers more advantages compared to embryo freezing, such as fertility preservation in women at risk of losing fertility due to oncological treatment or chronic disease, egg donation, and postponing childbirth, and eliminates religious and/or other ethical, legal, and moral concerns of embryo freezing. In this review, the basic principles, methodology, and practical experiences as well as safety and other aspects concerning slow cooling and ultrarapid cooling (vitrification) of human embryos and oocytes are summarized.

1. Introduction

The first successful mouse embryo cryopreservation (CP) was reported independently from each other by two research groups in 1972 [1–3]. One year later, the birth of the first calf from frozen embryo was published [4]. The first human pregnancy from frozen embryo was achieved with the same procedure used successfully for CP of mouse and cow embryos; however, it was terminated by spontaneous abortion in the 2nd trimester [5]. Since then, both sperm and embryo CP have become routine procedures in human assisted reproduction (AR) and oocyte CP is being introduced into clinical practice and is getting more and more widely used.

Embryo CP has decreased the number of fresh embryo transfers and maximized the effectiveness of the IVF cycle. Similarly, embryo CP is a crucial tool in cases of cancelled embryo transfer (ET) due to ovarian hyperstimulation risk, endometrial bleeding, elevated serum progesterone levels on the day of triggering, or any other unplanned events. There is still a large debate on the best stage, protocol/procedure, and cryoprotective additives (CPA) to use. The average potential

of a frozen stored embryo to become a living child lies in the order of 4%, and babies born from cryopreserved embryos do not represent more than 8–10% of the total number of babies born from AR [6]. However, it is unquestionable that successful CP of zygotes/embryos has greatly enhanced the clinical benefits and cumulative conception rates possible for couples following a single cycle of ovarian stimulation and IVF. Results expressed as the augmentation of the delivery rate per oocyte harvest vary greatly in the literature, between 2% and 24% [7]. The data shows that women who had transfers of fresh and frozen embryos obtained 8% additional births by using their cryopreserved embryos [8, 9].

The metaphase II (MII) oocyte has a very special structure (i.e., large size, very sensitive to low temperature, extremely fragile, high water content, low surface to volume ratio, presence of the spindle and other cell organelles, not optimal plasma membrane permeability to CPA and water, etc.) that leads to complex difficulties associated with its CP. The spindle is crucial for the events following fertilization in the completion of meiosis, second polar body formation, migration of the pronuclei, and formation of the first mitotic spindle. The damage (depolymerization) and/or absence of

the spindle compromise the ability of the oocyte to fertilize and undergo normal preimplantation development. In addition, hardening of the zona pellucida—which is a consequence of CP—can adversely affect the normal fertilization process. However, oocyte CP offers more advantages compared to embryo freezing: (1) fertility preservation in women at risk of losing fertility due to oncological treatment, premature ovarian failure, or chronic disease; (2) it can help alleviate religious and/or other ethical, legal, and moral concerns of embryo storage; (3) it helps to overcome problems such as when the husband is unable to produce a viable sperm sample or when spermatozoa cannot be found in the testis at a given moment in case of nonobstructive azoospermia; (4) it makes “egg banks and/or egg donations” possible by eliminating donor-recipient synchronization problems; and (5) it allows women to postpone childbirth until a later time/age (e.g., after establishing a career, etc.). The latter is called social freezing when the oocytes are cryopreserved for nonmedical purposes. For about 10 years, in parallel with the technical improvement of oocyte freezing, the possibility of egg storing for nonmedical purposes is more extensively discussed and more commonly accepted by the general population and expert committees in the USA and Europe. The aim of the social freezing is to prevent age-related fertility decline which is widely promoted by fertility centers and the lay (unacademic) press throughout the world. It is a fact that the best reproductive performance/ability of women is around their 25–30 years of age. Afterwards pregnancy rates decline relatively fast from 35 years and miscarriage rates rise exponentially. After the age of 43 years, chances of becoming pregnant are very low [10, 11]. However, it is a worldwide tendency that women decide to give birth in their elder ages, as compared to earlier/20–30 years ago. Data of our patients having frozen cycle indicate that the average age ($n = 3601$) increased from 31.8 to 35.4 in the last 10 years (Figure 1).

In the case of almost 70% of the frozen cycles the patients were between 31 and 40 years old and 7.5% of them were >41 (Figure 2).

The “age effect” is detectable in the frozen embryo survival rate which slowly but continuously decreased in the last 10 years as the average age of the patients increased by 4 years without doing any modification in the freezing process (89% versus 81%; $P < 0.0001$). The number of successful frozen cycles is significantly lower over 30 years and there is a strong significant difference over 35 compared with under 30 years of age ($P < 0.01$ and $P < 0.0002$). The success rate of embryo/oocyte CP depends on several variables: efficacy of the freezing process, carriers used for vitrification (open versus closed), frequency of cycles with CP in the assisted reproductive program, the criteria for selection of embryos/oocytes for freezing, and the results of fresh embryo transfers. Results can be expressed as survival rates (but it is not enough alone, retention of normal physiological function of the cell organelles is essential), implantation rates, pregnancy rates, or delivery rates per transferred or thawed embryos or harvested oocytes [12].

In this review, we summarize recent results including our own experiences concerning oocyte and embryo CP.

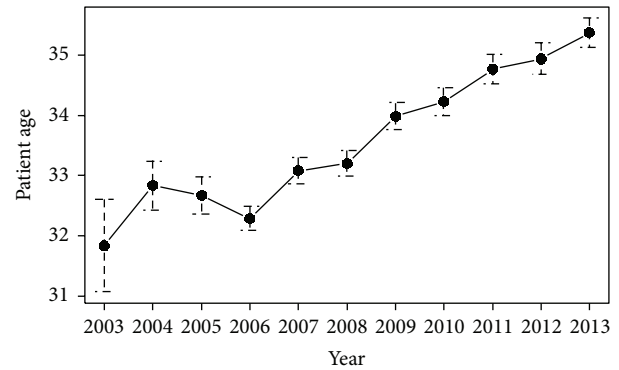


FIGURE 1: Increasing of average patient age in the last decade. Data are presented as mean ± SE.

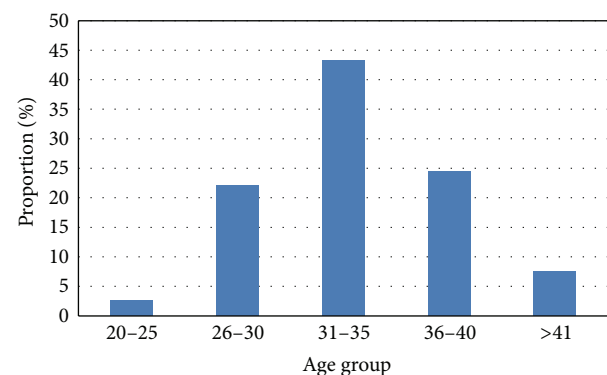


FIGURE 2: Proportion of age groups at our clinic.

2. A Short Overview of the Basic Principles and Methodology of Slow Cooling and Vitrification

The traditional slow cooling methods for CP are referred to as *equilibrium cooling*, and the rapid/ultrarapid procedures (vitrification) as *nonequilibrium cooling* [13–15]. Various factors influence the survival of embryos and oocytes cryopreserved by equilibrium or nonequilibrium cooling procedures [8, 16].

3. Traditional Slow Cooling of Embryos and Oocytes

The greatest challenge during the CP of embryos and oocytes is to prevent the formation of ice crystal and toxic concentrations of solutes, which are the two main causes of cell death associated with CP, while maintaining the functionality of intracellular organelles and the viability of the embryo/oocyte. In order to do so the freezing solution, in which the cells are suspended, must be supplemented with cryoprotective additives (CPA). Exposure to CPA supports the dehydration of the cell and reduces intracellular ice formation. The CPA may be divided into two groups: *intracellular/membrane-permeating* (i.e., propylene glycol/PG/, DMSO, glycerol/G/, and ethylene glycol/EG/) and *extracellular/membrane-nonpermeating* compounds (i.e.,

sucrose, trehalose, glucose, amid, ficoll, proteins, and lipoproteins). The permeable CPA displaces water via an osmotic gradient and partly occupies the place of the intracellular water, while the extracellular CPA increases the extracellular osmolarity generating an osmotic gradient across the cell membrane supporting the dehydration of the cell before CP. At the same time, it prevents the rapid entry of water into the cell after thawing during rehydration/dilution out of the permeating CPA [8, 13–15].

Dehydration of the cell mainly depends on the permeability properties of the cell membrane. There are differences in permeability among the embryos of different species to water and permeating CPA. Embryos usually are less permeable to G than to PG or EG. Furthermore, the earlier the stage of development, the less permeable are the embryos [15–17]. The permeability properties of immature and mature oocytes differ and can vary by 7-fold between individual human MII oocytes [18, 19]. This difference in membrane permeability may have a strong impact on the outcome of slow freezing of oocytes but can be controlled by the elevation of the concentration of the nonpermeable CPA and the environmental temperature [20, 21]. By having the concentration of nonpermeating CPA increased (sucrose: 0.2 and 0.3 M) higher survival rates were reported, and the overall fertilization rates of frozen-thawed oocytes appeared to be similar to those of fresh oocytes [20, 22–28].

Prior to slow cooling, dehydration of the embryos/oocytes is carried out by exposure to a mixture of permeable and nonpermeable CPA (duration: 10 minutes). In the case of human embryos/oocytes, with very few exceptions, low concentration of PG (1.5 M) and sucrose (0.1–0.25–0.5 M) is used for early cleavage stage embryos and oocytes and G for blastocyst stage embryos. In case of the original successful CP protocol mouse and cow embryos were cooled with a slow cooling rate (between minus 0.3°C–0.5°C/min) to very low temperatures of minus 80°C–120°C [1–5]. Therefore, the duration of the procedure was very long (several hours). Willadsen [29] and Willadsen et al. [30] described a variation of this method in which sheep and bovine embryos were cooled slowly at a rate of 0.3°C/min, but only to minus 30–35°C before being plunged into liquid nitrogen (LN₂) [29, 30]. With this modification the duration of the CP process was dramatically shortened (1.0–1.5 hours). Since then, this short protocol has become the treatment of choice for freezing of domestic animal embryos. Despite the excellent results achieved with animal embryos, human embryos are generally frozen with a low cooling rate of 0.3°C/min to about minus 30°C to 40°C, followed by an increased cooling rate of minus 50°C/min to a temperature of minus 80°C–150°C before being plunged into LN₂ [7, 8]. During slow cooling, the dehydration process is thought to continue until minus 30°C, after which any remaining water is super cooled [14]. During the slow cooling phase ice nucleation (seeding) is induced manually between –5 and –8°C (close to the true freezing point of the solution).

Embryos/oocytes cooled slowly to subzero temperatures of minus 30°C to 40°C before being rapidly cooled to minus 196°C require rapid warming/thawing in warm water of 25°C–37°C [13, 17].

Rapid thawing is followed by removal of the CPA from the embryo/oocyte. Rehydration of the cells is carried out in decreasing concentrations of permeating CPA, generally in the presence of increased concentrations of nonpermeating CPA. A common practice is to dilute CPA out of the frozen embryo/oocyte in a stepwise fashion. The use of a nonpermeating solute, such as sucrose as an osmotic buffer, decreases the chances of an osmotic shock and shortens the duration of the process (see earlier) [8, 16, 27, 31, 32]. Long term storage of embryos and oocytes requires temperatures below minus 130°C, the glass transition temperature of water. In practice, the easiest and safest way is to store cryopreserved embryos/oocytes in LN₂ at minus 196°C. Mouse model experiments indicate that the extended storage of embryos/oocytes does not affect the outcome of thawed cycles [17]. Live mice and sheep have been produced from cryopreserved embryos stored for more than 15 years in LN₂ [17]. Children have been born from embryos that were cryopreserved for more than 8 and 12 years [33].

4. Vitrification (Ultrarapid Cryopreservation) of Embryos and Oocytes

Vitrification (i.e., a glass-like state) is an alternative approach to embryo/oocyte CP which has been recently described as a revolutionary technique; however, the first successful embryo vitrification was published in the middle of the 1980s [34]. Vitrification is different from slow freezing in that it avoids the formation of ice crystals in the intracellular and extracellular space [34]. Vitrification is the solidification of a solution by an extreme elevation in viscosity at low temperatures without ice crystal formation, a process achieved by a combination of a high concentration of CPA (4–8 mol/L) and an extremely high (ultrarapid) cooling rate [15, 35–37]. In contrast to slow freezing (when dehydration of the embryos/oocytes starts during the equilibration in the freezing solution prior to slow cooling and continues during slow cooling to minus 30–35°C), during vitrification, cells are dehydrated mainly before the start of the ultrarapid cooling by exposure to high concentrations of CPA, which is necessary to obtain a vitrified intracellular and extracellular state afterwards. The potential risk associated with the vitrification procedure is the high concentration of CPA that could be toxic to cells. However, it is possible to limit CPA toxicity by mixing different CPA, thereby decreasing the relative concentration of each CPA, and by reducing the exposure time of embryos/oocytes to the solution to a minimum [15, 34]. The freezing solutions that are commonly used for vitrification are composed of permeating (e.g., EG, G, DMSO, PG, acetamide; >4 M) and nonpermeating (e.g., sucrose, trehalose; >0.5 M) agents. In some protocols, the vitrification medium is also supplemented with macromolecules such as polyethylene glycol, ficoll, or polyvinylpyrrolidone [15, 34, 37]. By increasing viscosity, the macromolecules support vitrification with lower concentrations of CPA. In order to further increase the cooling rate (>10,000°C/min) necessary for successful vitrification, the volume of the solution in which the embryos/oocytes are vitrified has been recently

dramatically decreased (0.1–2 μ L). To achieve this, special carrier systems (open versus closed) have been developed such as open pulled straws, Flexipet-denuding pipettes, Cryotop, electron microscopy copper grids, cryoloops, or the “Hemi-Straw” system [15, 35, 37, 38]. Closed systems have been developed for safety reasons. Comparing the open and closed systems Bonetti et al. [39] using closed carriers reported acceptable survival rates, but with multiple vesicles throughout the cytoplasm of oocytes which can be a likely consequence of not rapid enough temperature reduction in the closed system [39]. However, because of the improving results, the application of vitrification—especially for CP of human blastocyst and oocyte—has recently been greatly increased [15].

Technically vitrification is very difficult to perform, because of the very concentrated, viscous, and small volume of vitrification solutions in which the embryos/oocytes must be handled for only a very limited amount of time (<1 min) prior to and during vitrification. Therefore, in order to achieve the optimal/high survival rate the embryologist performing vitrification has to be very well trained. This is not the case in the case of slow freezing when the embryos/oocytes are cooled slowly (with a special cell freezer), because slow freezing is a more flexible technique. Similarly to slow freezing, rapid thawing is required for the optimal survival of vitrified embryos/oocytes, followed by stepwise rehydration using similar techniques employed after slow cooling.

5. Practical Experiences with Human Embryo Cryopreservation Using Slow Cooling and Vitrification

Generally, PG is used for the freezing of zygote and cleavage stage embryos and G for the CP of blastocysts [7, 8, 12, 35]. For many years, the preferred stages for human embryo CP were the zygote and early cleavage stages. Blastocyst freezing was abandoned for years, since only 25% of zygotes were able to reach the blastocyst stage in vitro in usual culture media, and overall low pregnancy rates were reported. Recently, new embryo culture systems—such as the coculture on feeder cells and the sequential media—have been developed making it possible to obtain good quality blastocysts in 50–60% of the cases [40]. Therefore, the importance of blastocyst CP increased in the last 8–10 years. Furthermore some of the published data indicate that human blastocysts obtained using sequential media appear to be only half as cryoresistant as the cocultured ones [7, 40–42].

Early cleavage stage embryos are considered surviving CP when they keep at least half of their initial blastomeres intact after thawing. The moderate loss of cells did not significantly influence implantation. In an early, large multicentre study with 14 000 cleavage stage slow frozen and thawed embryos it was determined that 73% of the embryos had at least half of their initial blastomeres still intact and the results showed clinical pregnancy and implantation rates of 16 and 8.4%, respectively, after transfer. In another study of over 300 single frozen embryo transfers of Day 2 embryos at the 4-cell stage and the embryos lost only a single blastomere during

freezing/thawing (25%) similar implantation equivalent with fully intact frozen embryos and also with fresh embryos was obtained [25]. Data obtained from experience with slow cooling in 1.5 M PG plus 0.1 M sucrose is that around 75–85% of all cryopreserved cleavage stage embryos survive CP and that 50 to 60% of all thawed embryos will be totally preserved (100% of blastomeres survived). The lower survival rate of biopsied cleavage stage embryos could be improved by increasing the concentration of the nonpermeating CPA, sucrose prior to freezing [43]. Edgar et al. [44] observed that increasing the concentration of the sucrose from 0.1 M to 0.2 M resulted in a highly significant increase in survival [44]. Not only did the survival rate increase but the proportion of the fully intact embryos also significantly increased (54.6% versus 80.5%). The implantation rate per embryos thawed increased too, but it was not as significant (22.1% versus 17.5%). This modified slow freezing technology together with increased sucrose concentration has produced results which are equivalent to that of the best results obtained with vitrification.

The most widely used freezing solution for slow cooling of blastocysts is the combination of G and sucrose. The reported survival rates with a minimum of 50% survival of the inner cell mass and trophoblast cells are around 69%–98%, and the implantation rates are around 16%–30% [40–42]. Data indicates that the speed of development has influence to the survival rate. Reexpansion of frozen-thawed blastocysts in vitro is considered to be a very good sign of survival (70 to 80% of thawed blastocysts). A survival rate of 88% was reported for slow cooled blastocysts, whether or not they had been biopsied for PGD. In the same study the implantation rate was similar for fresh (34%) and thawed (35%) PGD blastocysts. Based on more than 400 frozen-thawed embryos Konc et al. [45] found no difference in the survival, implantation, and pregnancy rates of embryos cryopreserved on Days 3 and 5. However, in the pregnant group significantly higher implantation rate was observed with Day 5 blastocyst than with Day 3 embryos [45].

Early cleavage stage human embryos have been successfully vitrified in DMSO, EG, DMSO + sucrose, EG + sucrose and DMSO + EG + sucrose based solutions, and cca. 60%–80% of survival rate with at least 50% of their original blastomeres intact, and several pregnancies/deliveries have been reported (pregnancy rate: 10%–15%) [15, 46]. Kuwayama et al. [47] vitrified cleavage stage embryos with EG + DMSO + sucrose and the results showed a small but significant increase in survival (98% versus 91%), but no difference in the pregnancy rate relative to slow cooling was found [47]. In a similar comparative study no difference was found in the survival and implantation rates between slow cooling and vitrification [48]. Balaban et al. [49] using PG + EG + sucrose based solution observed higher survival (94.8% versus 88.7%) and a higher rate of fully intact embryos (73.9% versus 45.7%) in the vitrified group, compared with slow frozen Day 3 embryos which had been frozen in 1.5 M PG + 0.1 M sucrose [49]. The use of special carrier systems—through increased cooling speed—resulted in better survival and pregnancy rates after vitrification (survival rate of 90% and pregnancy rates of 25–60%). Kolibianakis et al. [50] in their review

concluded that vitrification was not associated with a higher probability of pregnancy than slow freezing in experienced groups, but it did show a higher postthawing survival rate in cleavage and blastocyst stage embryos [50].

For blastocyst vitrification the most widely used solution is a mixture of EG and DMSO. Blastocysts have recently been successfully vitrified with improved survival rates in different carrier systems allowing ultrarapid cooling in small volumes of CPA solution. The reported overall survival rates are around 70–99% and the implantation rates are around 20–50% [51–55]. Ebner et al. [56] having used closed system reported 74% survival and 39% implantation rates [56]. With another closed system the overall reported survival rate was 78%, with 56% of blastocysts fully intact after thawing. The implantation rate of the fully intact blastocysts was 16% compared to 6.4% in those with moderate damage [57]. Vanderzwalmen et al. [58] published 86% survival rate and an implantation rate of 30% having used an aseptic vitrification system [58].

In a comparative study Kuwayama et al. [47] found that the survival of vitrified blastocysts was slightly but significantly higher (90%) than that of slow cooled blastocysts (84%). However, pregnancy rates (53% versus 51%) and live birth rates (45 versus 41%) per transfer were not significantly different [47]. In a study with over 500 blastocysts in each group, Liebermann and Tucker [60] obtained no difference in the survival rate (96.5% versus 92.1%), in the pregnancy rate per transfer (46.1% versus 42.9%), and in the implantation rate (30.6% versus 28.9%) between vitrified and slow frozen groups [60].

6. Practical Experiences with Human Oocyte Cryopreservation Using Slow Freezing or Vitrification

Since the first successes achieved in the field of human oocyte CP many changes have been introduced into the slow cooling procedure. Increasing the sucrose concentration both in the slow freezing and vitrification solutions (from 0.1 M to 0.3 M) increased the rate of dehydration and the survival and fertilization rates of MII oocytes in a dose-dependent manner [20, 22–28]. Changing the temperature of the equilibration with CPA, ice nucleation (seeding) and plunging embryos into LN₂, replacing sodium with choline (low sodium medium), or injecting sucrose directly into the cytoplasm of the oocyte all improved oocyte survival [32, 61, 62]. These results indicate that there is still room to improve the outcome of slow freezing of oocytes.

Slower development relative to fresh controls, both with respect to timing of the first cleavage division and the developmental stage reached on Day 2, has been observed in oocytes slowly cooled in 0.3 M sucrose [24, 63]. Konc et al. [22] reported comparable fertilization rates (fresh: 83%; frozen: 76%) but significantly slower development in the cryopreserved group, although implantation rates per embryo and oocyte were similar (fresh: 18% and 11%; frozen: 15% and 7%) [22]. Their results show a very pronounced difference in the cell stage on Day 2 between the frozen and

fresh groups of oocytes ($P < 0.05$) as they found slower embryo development in the frozen oocyte cycles relative to fresh cycles. In the frozen group 64% of the embryos remained in the 2-cell stage and only 17% were in the 4-cell stage on Day 2. In contrast, in the fresh group on Day 2 66% of embryos were already in 4-cell stage and only 25% of them were in the 2-cell stage. Oocytes analyzed immediately after thawing displayed severe disorganization or disappearance of the spindle after slow freezing or vitrification. However, culturing oocytes for 1 to 3 hours after CP allows the spindle to repolymerize [11, 64–67]. Martinez-Burgos et al. [67] observed that vitrification seems to maintain the spindle apparatus at higher rates; therefore vitrified oocytes tend to repolymerize their spindles more effectively and faster than slow cooled oocytes; however, they showed higher misalignment between the meiotic spindle and the polar body [67]. Interestingly, they found no differences in DNA fragmentation between slow cooling and vitrification. Ciotti et al. [68] also reported that spindle recovery was faster in vitrified oocytes compared to slow cooled ones [68]. In contrast, Cobo et al. [64] found comparable spindle recovery from vitrification and slow freezing after 3 hours of incubation [64]. Konc et al. [70] investigated the spindle dynamics/displacement in frozen-thawed human oocytes. In each oocyte, prior to freezing and after thawing and 3 hours in vitro culture—just prior to ICSI—the presence and location of the spindle was determined with Polscope. Their results indicate that by observing the response of the individual oocytes the spindle does not always reform in its original position within the oocyte. After thawing and culturing the oocytes, they were able to visualize the spindle in 84.3% of the oocytes. However, they found that in half of the oocytes (53.1%) in which the spindle was rebuilt/visualized it was detected in a new location, not at the initial place, indicating that the spindle and the polar body move relative to each other [70].

The most widely used vitrification solution consists of a mixture of permeating (2.7 M EG and 2.1 M DMSO) and nonpermeating CPA (0.5 M sucrose). New data obtained with the improved vitrification techniques (i.e., decreased volume of vitrification medium and very rapid cooling speed) show an increase in the postthaw survival and fertilization rates of vitrified human oocytes which are comparable to the fresh control oocytes. Cobo et al. [71] published their findings from a randomized controlled trial of over 3000 fresh and 3000 vitrified oocytes (92.5% survival) in an oocyte donation program, confirming no detrimental effects of vitrification on subsequent fertilization, development, or implantation [71]. Others using the same vitrification protocol, also in oocyte donation programs, reported similar outcomes [72, 73]. Results obtained with the same technique in standard infertility programme showed a trend towards lower overall clinical outcomes from vitrified oocytes, especially over the age of 34 [74–76].

Comparing the results of slow freezing and vitrification we have to take into consideration that most of the published data generated by oocyte vitrification was obtained mainly by open systems and from oocyte donation programmes

in which the egg donors were fertile and generally young women.

7. Safety and Other Aspects of Oocytes and Embryo Cryopreservation

The total number of children born worldwide after the fertilization of frozen and thawed oocytes is more than 1500 [77–79]. Studies indicate that pregnancies and infants conceived after oocyte CP do not present with increased risk of adverse obstetric outcomes or congenital anomalies [80]. No increase in the number of abnormal or stray chromosomes has been observed in the thawed oocytes [81]. In addition, no difference was found when comparing the incidence of chromosomal abnormalities in human embryos obtained from fresh and frozen oocytes [81, 82]. The follow-up study of 13 children born from frozen oocytes failed to reveal any abnormalities in karyotype or organ formation, mean age at delivery, and mean birth weight [83]. In another study no intellectual and/or developmental deficits were found in children conceived from cryopreserved oocytes [69, 83–85]. Despite the promising results, there are still concerns regarding the possibility of chromosomal aneuploidies or other karyotypic abnormalities, organ malformations or other developmental problems in offsprings; therefore, further follow-up studies with adequate numbers of patients involved are needed to clarify this very important question.

For patients, who are facing infertility due to chemotherapy/radiotherapy, oocyte CP is one of the few options available to keep their fertility potential [78, 86]. Thus, the standpoint of the Practice Committee of the Society for Assisted Reproductive Technology, the Practice Committee of the American Society for Reproductive Medicine, and the American Society of Clinical Oncology is that (1) oocyte CP holds promise for future female infertility preservation, (2) recent laboratory modifications have resulted in improved oocyte survival, fertilization, and pregnancy rates from cryopreserved oocytes, (3) no increase in chromosomal abnormalities, birth defects, or developmental deficits has been noted in the children born from frozen oocytes, and (4) oocyte CP should not be considered any more as an experimental technique and must be recommended to cancer patients only and carried out with appropriate informed consent.

At present, spermatozoa and embryos/oocytes are commonly frozen/stored in LN₂ using straws/vials and newly developed open or closed carriers used for vitrification. Since the freezing container may leak or shatter during freezing, the potential for contamination of liquid nitrogen represents a real danger, especially in case of the “open carriers” developed for embryo/oocyte vitrification with ultrarapid cooling. The occurrence of cross-contamination during LN₂ storage of biological material and subsequent cross-infection of patients has previously been demonstrated [87]. Viruses have previously been found to survive direct exposure to LN₂, including vesicular stomatitis virus, herpes simplex virus, adenovirus, and papilloma virus [88]. There is also evidence of contamination of LN₂ by other microorganisms,

including a wide range of bacterial and fungal species [89]. Given the strength of the evidence of LN₂ contamination by microbes and cross-infection in certain situations the possibility of contamination or cross-contamination during reproductive cell CP should be taken seriously. There are a number of relatively simple details and possible changes to CP procedures that can minimize the potential for contamination or cross-contamination of stored samples; for example, all patients and donors whose reproductive cells will be cryopreserved should be screened (e.g., HBV, HCV, HIV, etc.); it is highly recommended that the infected materials be stored in separate containers for each infection; instead of open systems, closed systems should be used for vitrification; finally, the storage container should be periodically emptied and cleaned [87, 90, 91]. However, in a comparative study all embryos cryopreserved in sealed straws and cryovials were free from viral contamination [87]. Transport of material vitrified in very small volumes may also raise questions related to its impact on survival [91].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Recent Progress in Cryopreservation of Bovine Oocytes

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Principle of oocyte cryoinjury is first overviewed and then research history of cryopreservation using bovine oocytes is summarized for the last two decades with a few special references to recent progresses. Various types of cryodevices have been developed to accelerate the cooling rate and applied to the oocytes from large domestic species enriched with cytoplasmic lipid droplets. Two recent approaches include the qualitative improvement of IVM oocytes prior to the vitrification and the short-term recovery culture of vitrified-warmed oocytes prior to the subsequent IVF. Supplementation of L-carnitine to IVM medium of bovine oocytes has been reported to reduce the amount of cytoplasmic lipid droplets and improve the cryotolerance of the oocytes, but it is still controversial whether the positive effect of L-carnitine is reproducible. Incidence of multiple aster formation, a possible cause for low developmental potential of vitrified-warmed bovine oocytes, was inhibited by a short-term culture of the postwarm oocytes in the presence of Rho-associated coiled-coil kinase (ROCK) inhibitor. Use of an antioxidant α -tocopherol, instead of the ROCK inhibitor, also supported the revivability of the postwarm bovine oocytes. Further improvements of the vitrification procedure, combined with pre- and postvitrification chemical treatment, would overcome the high sensitivity of bovine oocytes to cryopreservation.

1. Introduction

Many reproductive biotechnologies have been applied to efficient production of large domestic animals, such as pigs and cattle with the high economic importance. In cattle, those originally developed in the decades of 1950s to 1970s include artificial insemination and multiple ovulations/embryo transfer combined with or without cryopreservation of spermatozoa [1] and preimplantation-stage embryos [2, 3], respectively. Successful cryopreservation of spermatozoa and embryos made these technologies more practical and available for commercial use, because of their potential advantages to allow long-distance transportation and to omit estrous synchronization, thus reducing the number of recipient female population to be maintained. Thereafter, embryo production by in vitro maturation (IVM) and in vitro fertilization (IVF) using immature oocytes from abattoir-derived ovaries and frozen-thawed spermatozoa became more or less routine since the decade of 1980s [2, 3],

and production of cloned embryos has been promising with the progress of somatic cell nuclear transplantation [4].

Cryopreservation of unfertilized oocytes can be combined with these advanced reproductive technologies, in addition to its potential advantage as oocyte banking for preserving female genetic resources. Revivability of cryopreserved oocytes from small rodents and humans is extremely high, adapting well to the maintenance of the huge numbers of gene-modified transgenic strains and the efficient use in therapies for human infertility [5, 6]. However, in bovine species, the low fertilization rates and developmental competence of cryopreserved oocytes still need to be improved. Some review articles regarding this topic are available in cattle [7, 8] and pigs [8, 9]. In this paper, principle of oocyte cryoinjury is first overviewed. Then, research history of cryopreservation using bovine oocytes is summarized for the period from 1992 to 2013 with a few special references to very recent progress.

2. Basic Cryobiology for Oocytes

2.1. Events around Fertilization. Once oocytes resume the first meiotic division, the nuclear envelop (germinal vesicle: GV) is disintegrated, allowing the nuclear material to mix into the cytoplasm. Some alterations also occur in organelles such as mitochondria, cytoskeleton, and cortical granules. Microfilaments of actin are involved in cell shape modifications and movements, and microtubules (cylindrical bundle, composed from heterodimer of α - and β -tubulin) form the spindle apparatus [10]. A spermatozoon has a pair of distinct centriolar structures as the proximal centriole located within the connecting piece under the sperm head and the distal centriole organized vertically to the proximal counterpart and aligned with the sperm tail [11]. During fertilization in most mammalian species including cattle, spermatozoal centrosome, composed from the two centrioles and the pericentriolar materials such as γ -tubulin, centrin, and pericentrin, is brought into an oocyte. The centrosome plays a critical role in assembly of the microtubule network (sperm aster) that brings both male and female pronuclei to the center of the newly formed zygote [12]. Thus, the centrosome is considered to be the microtubule-organizing center (MTOC), with duplication during the pronuclear stage and the subsequent separation to serve as mitotic centers anchoring the chromosomes during the first cleavage [13, 14]. Abnormalities of the spindle/MTOC function/sperm aster have been shown to directly correlate with the loss of developmental potential after IVF, because they are crucial for completion of the second meiosis, extrusion of the polar body, migration of the pronuclei, and formation of the first mitotic spindle [15].

2.2. High Cryosensitivity of Oocytes. Biological activity is completely stopped at very low subzero temperature, and the cell viability and functional state may be preserved for long terms [16]. However, some physical stresses can damage cells at the various subzero temperatures. Intracellular ice formation is one of the biggest causes to cell damage; hence, the freezing protocols use a combination of dehydration, freezing point depression, supercooling, and intracellular vitrification in an attempt to avoid cell damages [17]. Therefore, it is important to use cryoprotective additive (CPA), such as dimethyl sulfoxide (DMSO), ethylene glycol (EG), or glycerol alone or in combination, when cryopreserving cells in any methods. Due to both of hydrophobic and hydrophilic characteristics, as well as the relatively small molecular weight, these CPAs are permeable to the plasma membrane. On the other hand, use of CPA induces some adverse effects such as osmotic injury and toxicity of the CPAs.

Incidence of cryoinjuries depends on the size and shape of the cell, the permeability of the cell membranes, and the quality of the cells. However, these factors differ from species, developmental stage, and origin [18]. Although offspring has been born using frozen-thawed oocytes from various species, the ability to support embryo development following cryopreservation procedures is still low. This may be attributed to the susceptibility of oocytes to damage during cooling and/or freezing and subsequent thawing because of their complex structure. Unfertilized oocytes are much larger than

the blastomeres of an early embryo and therefore have a small surface to volume ratio [13]. This led to dehydration and penetration of CPA being difficult to achieve, which attributes to the difficulty in cryopreservation. Furthermore, the plasma membranes of oocytes differ significantly from those of embryos, partially due to the lack of aquaporin expression which affects the movement of water and CPAs [19]. There is a rise of intracellular free calcium during fertilization, which makes the ionic strength and membrane potential of the plasma membrane [20]. Other adverse effects of cryopreservation procedures include the fracture damage in zona pellucida [21] and the destruction of intercellular coupling via gap junctions between cumulus cells and the oocyte [22, 23]. In general, the primary criteria to assess postthaw viability of oocytes are the presence or absence of membrane degeneration, cytoplasmic abnormalities and zona pellucida fractures [24]. Recent studies in humans have examined the meiotic spindle using a polarized microscope apparatus, which allows the visualization of the polymerization of the meiotic spindle after vitrification and warming. However, this technique is difficult in domestic animal species due to their high cytoplasmic lipid content, which hinders spindle examination. Therefore, such dark oocytes from domestic species must be typically examined through invasive methods, such as fluorescence microscopy and biochemical or molecular analyses [25].

Low fertilization rates of cryopreserved oocytes were reported to be associated with chilling and freezing injuries, including zona hardening due to premature release of cortical granules [22, 26] and spindle disorganization and loss or clumping of microtubules [27, 28]. Briefly, exposure of mature oocytes to CPA and/or chilling procedure induced the transient rise of intracellular free calcium and prevented the sperm entry via block mechanisms at the level of plasma membrane or zona pellucida [29–32]. These processes also result in damage to the meiotic spindle, actin filaments, chromosomal dispersal, and microtubule depolymerization [33, 34]. In addition, Hara et al. [35] proposed a third hypothesis for cryodamage of bovine oocytes that multiple aster formation frequently observed in vitrified-warmed and fertilized oocytes may be related to loss of ooplasmic function responsible for normal microtubule assembly. These possible hypotheses responsible for cryodamages of the oocytes are shown in Figure 1.

3. History

3.1. Learning from Embryo Cryoresearch. Knowledge can be concurrently accumulated from research history of embryo cryopreservation. Following the first successful freezing of mouse 8-cell stage embryos in 1972 [36], pregnancy from a cryopreserved cattle embryo was reported by Wilmut and Rowson [37]. These initial findings were then extended to embryos from several mammalian species including domestic animals [38–40] and human [41]. The protocol most commonly used for successful embryo cryopreservation at that time required slow cooling from upper -7°C to below -80°C in phosphate-buffered saline supplemented with DMSO or glycerol as a permeable CPA. During the slow

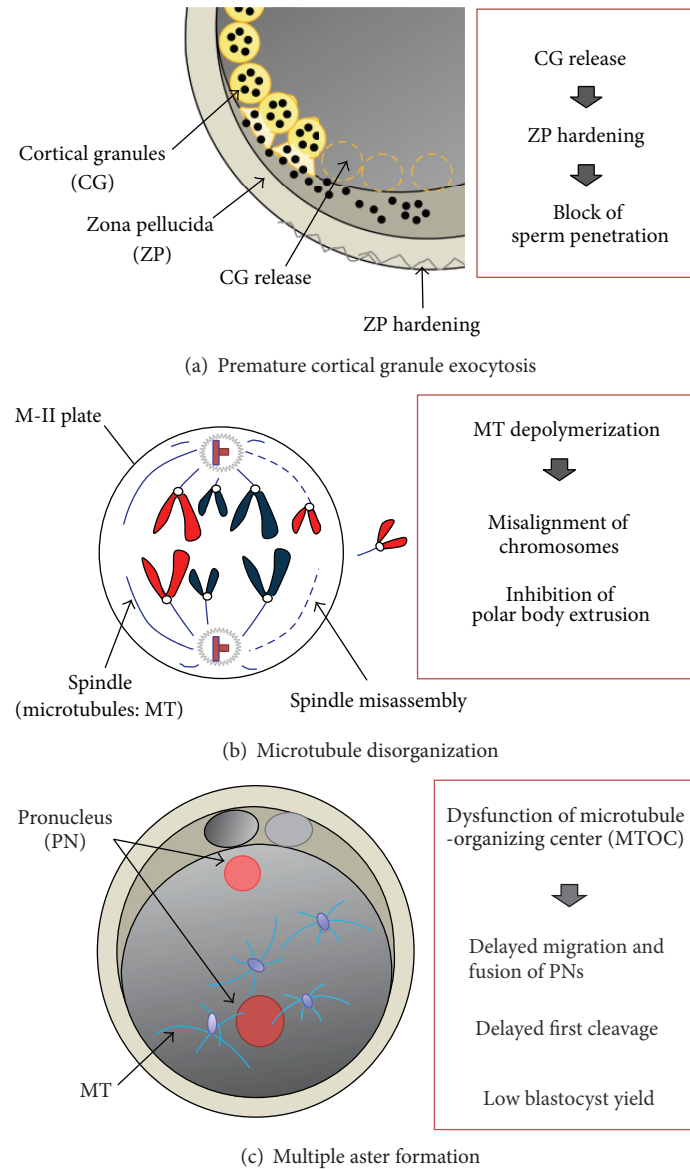


FIGURE 1: Hypotheses regarding cryoinjuries in mammalian oocytes. (a) Premature cortical granule exocytosis causes the hardening of zona pellucida, leading to block of sperm penetration. (b) Disorganization of microtubules means depolymerization of tubulin proteins, leading to misassembly of meiotic spindles, and subsequently resulting in misalignment of chromosomes and inhibition of the second polar body extrusion. (c) Multiple aster formation, resulting from ooplasmic dysfunction to support MTOC, is a possible cause of low blastocyst yield.

cooling, embryonic blastomeres are dehydrated in response to the osmotic pressure that gradually increases with the formation of extracellular ice crystals after ice seeding. The frozen embryos were warmed very slowly to avoid the rapid influx of extracellular water into the dehydrated cells during warming. This earlier protocol was labor-intensive and time-consuming.

In 1977, a two-step freezing method was reported using sheep and cattle embryos [42]. The slow cooling of embryos is interrupted at around -30°C to -36°C , followed by rapid cooling to -196°C . The embryos in LN_2 are believed to contain intracellular ice, although it is not detrimental at this point. But to survive, the frozen embryos must be warmed rapidly to avoid injury caused by recrystallization

of the intracellular ice. This two-step freezing regimen allows the development of a temperature-controlled, programmable freezer and is still used widely for many mammalian species. In cattle, pregnancy rates following transfer of embryos frozen in this way range from 50 to 60% [43]. Additional progress resulted from the use of EG as a CPA for embryos from domestic species. Using sucrose as an osmotic buffer, direct transfer of postthaw embryos into recipients without expelling them from the straws was reported by Leibo [44].

Then, a great breakthrough for simple and efficient cryopreservation has been reported by a very high cooling rate of fully dehydrated mouse embryos in highly concentrated solutes. Rall and Fahy [53] developed a novel approach to cryopreserve mouse embryos in 1985. This protocol involves

TABLE 1: A list on Day-2 cleavage and Day-8 blastocyst yield from bovine mature oocytes cryopreserved and fertilized in vitro.

Years	Method/device for cryopreservation	Oocyte cryosurvival		Literature
		Cleavage rate	Blastocyst yield	
1992	Freezing/French straw	42%	3%	Otoi et al. [45]
1992	Vitrification/French straw	22%	9%	Hamano et al. [46]
1996	Vitrification/EM-grid ^{*1}	40%	15%	Martino et al. [47]
1998	Vitrification/OPS ^{*2}	50%	13%	Vajta et al. [48]
2000	Vitrification/Microdrop	62%	11%	Dinnyes et al. [49]
2004	Vitrification/Cryotop	70%	7%	Chian et al. [50]
2005	Vitrification/GL-tip ^{*3}	49%	17%	Tominaga et al. [51]
2010	Vitrification/Cryotop	76%	12%	Zhou et al. [52]

^{*1}EM-grid: electron microscope grid; ^{*2}OPS: open-pulled straw; ^{*3}GL-tip: gel-loading tip.

dehydration of the embryos by exposing them to highly concentrated CPAs prior to cooling them to low temperature, rather than during the cooling process itself. The dehydrated embryos are rapidly cooled by being directly plunged into LN₂. Since the cryoprotective solution can be transformed into a stable glass without ice crystal formation during the rapid cooling process, this extremely rapid method of cryopreservation is referred to as “vitrification,” meaning “glass formation.” The application of vitrification as an alternative to conventional freezing can reduce the equipment required, but technician-dependent performance of vitrification process is the limited factor for its widespread use. So far, successful vitrification producing pregnancy and/or birth of live offspring has been reported with preimplantation embryos from various mammalian species including human. A wide variety of vitrification solutions and protocols have been employed even for the same type of embryo, that is, the same species and developmental stage.

3.2. Cryopreservation of Mature Oocytes. Cryopreservation of oocytes has short and less successful history when compared to the other reproductive cells as spermatozoa and embryos. The first successful IVF and birth of live offspring using frozen-thawed mouse oocytes was reported in 1976 by Parkening et al. [54], and followed by Whittingham [55] and Leibo et al. [56]. Other than the mouse, such a slow freezing procedure was acceptable for species whose oocytes are not sensitive to chilling, such as cat [57, 58] and human [59]. There are a few reports regarding successful pregnancies from frozen-thawed bovine oocytes [60, 61]. However, oocytes from the large domestic species are rich in cytoplasmic lipid droplets and very sensitive to chilling, resulting in the poor revivability following the slow cooling [62]. After the publication of innovative results by Rall and Fahy [53], vitrification has been attempted to apply to oocytes. Pregnancies or birth of live offspring have been published in mouse [63], human [64], and cattle [46], with an increased requirement for improving developmental competence of the vitrified-warmed oocytes.

In 1996, Martino et al. [47] reported that 15% of matured bovine oocytes developed into blastocysts following vitrification, under in vitro culture (IVC) conditions in which >40% of the non-treated fresh oocytes were able to develop

to that stage. That protocol, a pioneer work opening the new window for oocyte cryobiology, is characterized by the extremely rapid cooling rate of oocytes suspended in <1 μ L of a vitrification solution consisting of 30% EG plus 1.0 M sucrose placed onto electron microscope grids, a procedure derived from methods to cryopreserve *Drosophila* embryos [65]. The microgrids provide a cooling rate estimated to be <150,000°C/min, in contrast to 2,500°C/min with the conventionally used plastic straws. Vajta et al. [48] reported an alternative way of ultra-rapid cooling for vitrification of bovine oocytes. When the oocytes were aspirated with 20% EG and 20% DMSO solution into open-pulled straws (OPS) and cooled by directly plunging into LN₂, 13% of the post-warm oocytes could develop into blastocysts after IVF and IVC. The OPS method has been improved to use open-pulled glass capillaries [66, 67] or commercially available gel-loading tips [68] using different CPA combinations. Other types of cryodevices so far reported for ultra-rapid cooling are the “Cryoloop” [21] and “Cryotop” [69]. Complete containerless methods have also been reported from two independent laboratories [49, 70]. Blastocyst yields from frozen-thawed or vitrified-warmed bovine metaphase-II oocytes, reported during the last two decades [45–52], are summarized in Table 1. There was no significant improvement on the blastocyst yield from cryopreserved bovine mature oocytes (commonly exceeding 10%), even after increased cleavage rates as >60% by using different cryodevices and vitrification protocols were obtained.

3.3. Cryopreservation of Immature Oocytes. Cryopreservation of immature oocytes at the GV stage is also the subject for challenging endeavour. Vajta et al. [48] reported that 25% of bovine oocytes vitrified-warmed using OPS system could develop into the blastocyst stage on Day 8. While it is still unclear that the high revivability of post-warm GV oocytes in the OPS system is reproducible, birth of calves following transfer of embryos derived from cryopreserved immature oocytes [71] encouraged such challenges. Abe et al. [72] reported that 8% of bovine oocytes developed into blastocysts when they were exposed to EG + Ficoll + sucrose-based solution in a stepwise manner and vitrified-warmed on nylon-mesh holder as a cryodevice, with successful data on a live calf after transfer. Bovine oocytes at the GV

stage have homogenous (=less variable in size) lipid droplets that show little change following cooling, but intercellular coupling via gap junctions between cumulus cells and the GV-stage oocytes may be sensitive to osmotic stress. In addition, maintaining functional integrity of the cumulus cells after vitrification and warming is an important factor to harvest cytoplasmically-matured oocytes after subsequent IVM process.

4. Recent Improvement of Oocyte Cryosurvival

Our literature search failed to find recent papers published during 2011 to 2013 which described the significantly improved cryosurvival (blastocyst yield) of bovine oocytes by modifying the CPA composition, the cryodevice, or the CPA addition/dilution process in the vitrification procedures. Hence, a few chemical treatments of bovine oocytes during the IVM prior to vitrification and during the recovery culture after vitrification are highlighted in this section. Using mouse and porcine embryos, cellulose triacetate hollow fiber with a pore size of 7.5 nm has been proposed as a new device that can vitrify large amount of embryos without stepwise handlings of the embryos, reported recently by Matsunari et al. [73]. But this hollow fiber vitrification procedure has not yet been applied to the bovine oocytes.

4.1. Treatment During IVM. Large amount of cytoplasmic lipid droplets serves as energy resource but increases sensitivity of bovine oocytes to chilling injury during cryopreservation. Most of the lipid droplets locate at the periphery of plasma membrane or close proximal to organelles such as mitochondria and endoplasmic reticulum [74], both of which are the major target of cryodamage in oocyte organelles [75, 76]. Cytoplasmic lipid droplets can be partially removed from bovine oocytes by high magnitude centrifugation, and incidence of polyspermic penetration in the centrifuged and vitrified-warmed oocytes was significantly inhibited (blastocyst yield; 11% versus 7% in noncentrifuged control) [61]. It is also well known that vitrification of bovine oocytes induces mitochondrial dysfunction and loss of adenosine triphosphate (ATP) [77, 78]. L-Carnitine (Figure 2(a)), an active form of carnitine, can enhance lipid metabolism in animal cells and play an important role in the transportation of fatty acids from the cytoplasm to the mitochondria for β -oxidation [79]. Hence, L-carnitine can enhance ATP production in animal cells [80] and stimulate mitochondrial metabolism during maturation as firstly reported in mouse oocytes [81]. In porcine oocytes enriched with cytoplasmic lipid droplets, supplementation of L-carnitine into IVM medium reduced the amount of the lipid droplets and changed their distribution from the cortex to the medulla of oocyte cytoplasm [82]. Furthermore, supplementation of L-carnitine into IVC medium reduced the lipid content in bovine embryos and increased cryotolerance and developmental competence [83].

Two recent papers have described the effect of L-carnitine supplementation into IVM medium for bovine oocytes on their cryotolerance [84, 86], as summarized in Table 2. Both groups have employed Cryotop vitrification system for

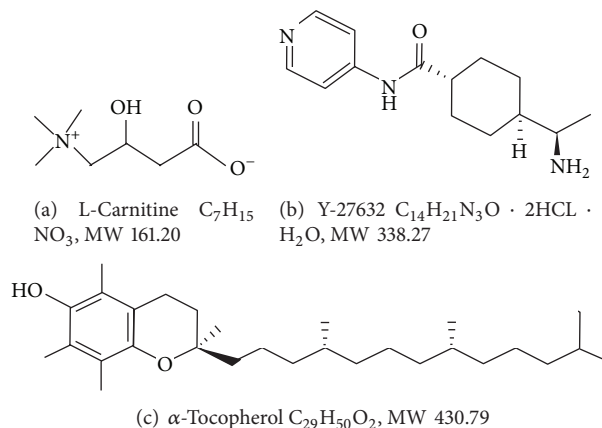


FIGURE 2: Structures of chemicals used for improvement of cryosurvival of bovine oocytes and resulted in significantly higher blastocyst yield in vitro [84, 85]. (a) L-carnitine, (b) ROCK inhibitor Y-27632, (c) α -tocopherol.

cryopreservation of bovine mature oocytes, but composition of the vitrification solution was different between the groups. Chankitisakul et al. [84] showed that bovine oocytes matured in the presence of 0.6 mg/mL L-carnitine had the higher developmental potential to blastocyst stage 8 days after vitrification and IVF when compared to those matured in the absence of the L-carnitine (34% versus 20%, fresh control; 44%). No significant changes were found in nuclear maturation rate, ATP content, timing of first cleavage, and blastocyst quality, while dislocation of lipid droplets from the peripheral area to inner cytoplasm was observed in the L-carnitine treated oocytes. On the other hand, negative result of L-carnitine treatment during IVM on cryotolerance of bovine oocytes has been reported by Phongnimitr et al. [86]. The attempt of this research group in Thailand seemed to be conducted almost simultaneously with the above Chankitisakul's group (based on the date of initial paper submission). Supplementation of 0.6 mg/mL L-carnitine to the IVM medium significantly improved the nuclear maturation rate (78% versus 68%) and the Day-7 blastocyst yield from non-vitrified control oocytes (31% versus 24%), but did not contribute to improve the cryotolerance of the oocytes (Day-7 blastocyst yield; 13% versus 11%). Further research would be needed to clarify the effect of L-carnitine on bovine oocytes.

Glutathione (L- γ -glutamyl-L-cysteinyl-glycine; GSH), a major nonprotein sulfhydryl compound, plays an important role in protecting cells against the destructive effects of reactive oxygen species (ROS) and regulating syntheses of DNA and proteins [87]. GSH level increases during oocyte maturation in the ovary and reaches a peak at the metaphase-II stage [88]. However, the GSH levels of IVM oocytes are lower when compared with those of ovulated oocytes, as reported in some species [89–92]. GSH synthesis in oocytes during IVM may be disturbed by a low availability of cysteine [87, 93]. Low molecular weight thiol compounds, such as β -mercaptoethanol and cysteamine, can promote cysteine (cystine) uptake through formation of a mixed disulfide compound [94, 95]. In addition, such thiol compounds supplemented into IVM medium can increase intracellular GSH

TABLE 2: Supplementation effect of L-carnitine to IVM medium on the cryotolerance of bovine oocytes [84, 86].

L-Carnitine	Vitrification	Nuclear maturation	Survival	Cleavage	Blastocyst yield
Chankitisakul et al. [84]					
–	–	67%	92% ^a	84% ^a	44% ^a
+	–	65%	93% ^a	84% ^a	45% ^a
–	+		81% ^b	57% ^b	20% ^b
+	+		83% ^b	63% ^b	34% ^a
Phongnimitr et al. [86]					
–	–	68% ^d	100%	78% ^c	24% ^d
+	–	78% ^c	100%	76% ^c	31% ^c
–	+		86%	67% ^d	11% ^e
+	+		88%	69% ^d	13% ^e

Superscripts a versus b, c versus d versus e in each column: $P < 0.05$.

Concentration of L-carnitine in the IVM medium: 0.6 mg/mL.

level and the developmental potential of the bovine oocytes [96]. It has been reported that GSH in bovine IVM-IVF oocytes can stimulate sperm aster formation [97]. Therefore, we have produced bovine IVM oocytes with 2.5-fold higher GSH content and then vitrified-warmed using Cryotop [98]. However, the high content of GSH in mature oocytes did not result in suppression of the high incidence of multiple aster formation (vitrified; 61% versus 53%, fresh control; 16% versus 17%) and improvement of developmental potential into Day-8 blastocysts (17% versus 16%, fresh control; both 41% regardless of thiol treatment for GSH).

4.2. Postvitrification Treatment. Generally, increased apoptosis in embryonic cells by oocyte vitrification procedure results in a decrease of developmental competence [99, 100]. Rho-associated coiled-coil kinase (ROCK), which is a kinase belonging to the AGC (PKA, PKG, and PKC) family of serine-threonine kinases, was realized as a downstream target of the small GTP-binding protein Rho [101], which could regulate growth, adhesion, migration, metabolism, and apoptosis of cells through controlling the actin-cytoskeletal assembly and contraction of cells [102]. Inhibition of the ROCK activity was involved in decrease of apoptosis in embryonic stem cell-derived neural cells [103]. Inhibition of the ROCK activity was also effective to improve the plating efficiency of dissociated human pluripotent stem cells after cryopreservation [104–108] and the revivability of in vitro produced bovine blastocysts after vitrification and warming [109].

Therefore, we used the ROCK inhibitor (Y-27632; Figure 2(b)) to improve the developmental competence of vitrified-warmed oocytes during 2 hours of recovery culture after Cryotop vitrification [85]. The vitrification solution consisted of 15% EG, 15% DMSO, and 0.5 M sucrose, and oocytes retrieved from 1-day-stored ovaries (10–12°C) were subjected to the IVM. As summarized in Table 3, treatment of the postwarm mature oocytes with 10 μ M Y-27632 resulted in significantly higher oocyte survival rate prior to the IVF, Day-2 cleavage rate, and Day-8 blastocyst yield (21% versus 14%, fresh control; 34%). The resultant blastocysts in Y-27632-treated group had better quality in terms of total cell number and apoptotic cell ratio. Time-dependent change in

mitochondrial activity of the vitrified-warmed oocytes was not influenced by ROCK inhibition during the period of recovery culture. However, the ability of ooplasm to support single-aster formation was improved by the ROCK inhibition (Figure 3(a)). Timing of first cleavage in the bovine oocytes vitrified-warmed and treated with Y-27632 was accelerated (Figure 3(b)), which may be favourable because bovine oocytes cleaving earlier are more likely to become blastocysts [110, 111]. Thus, inhibition of ROCK activity in vitrified-warmed bovine oocytes during short-term recovery culture could lead to higher developmental competence, probably due to decreased apoptosis and normalized function of the MTOC.

Using the same strategy, effect of two antioxidants, 10 μ M α -tocopherol (Figure 2(c)) or 250 μ M ascorbic acid, on rescuing vitrified-warmed bovine oocytes has been investigated in our laboratory. Oxidative stress by ROS must be one of the causes which may induce lipid peroxidation and/or organelle damage in bovine oocytes [112]. Interestingly, the supplementation of α -tocopherol, not ascorbic acid, to the recovery culture medium resulted in a significantly higher blastocyst yield from the postwarm oocytes as 37% versus 26% in the postwarm control oocytes (fresh control; 53%) (unpublished data of I. Yashiro and S. Hoshi). The improved baseline of blastocyst yield in the nonvitrified control group was due to the availability of the fresh (=within 6 h after slaughter) bovine ovaries for recent experiments.

5. Conclusion

Ultrarapid vitrification procedure, originally reported using electron microscope grid as cryodevice [47], has become a standard approach for cryopreservation of bovine oocytes with some modifications. Due to numerous efforts, as the development of novel cryodevice such as OPS [48] or Cryotop [69] and the preloading with low concentration permeable CPA [49, 70, 113], blastocyst yields at >10% have been commonly reported by several laboratories during the last decade. Two recent attempts to improve cryosurvival of bovine oocytes have been focused on; the qualitative improvement of IVM oocytes prior to the vitrification and

TABLE 3: Rescue of vitrified-warmed bovine oocytes with ROCK inhibitor (Y-27632) [85].

Vitrification	Y-27632	Survival	Cleavage	Yield	Blastocysts	
					Cell number	Apoptotic cell ratio
–	–	100% ^a	71% ^a	34% ^a	135.7 ^a	1.8% ^a
+	–	90% ^b	56% ^b	14% ^c	97.5 ^b	4.0% ^b
+	+	98% ^a	72% ^a	21% ^b	124.6 ^a	2.2% ^a

Superscript a versus b versus c in each column: *P* < 0.05.
Concentration of Y-27632 in recovery culture medium: 10 μ M.

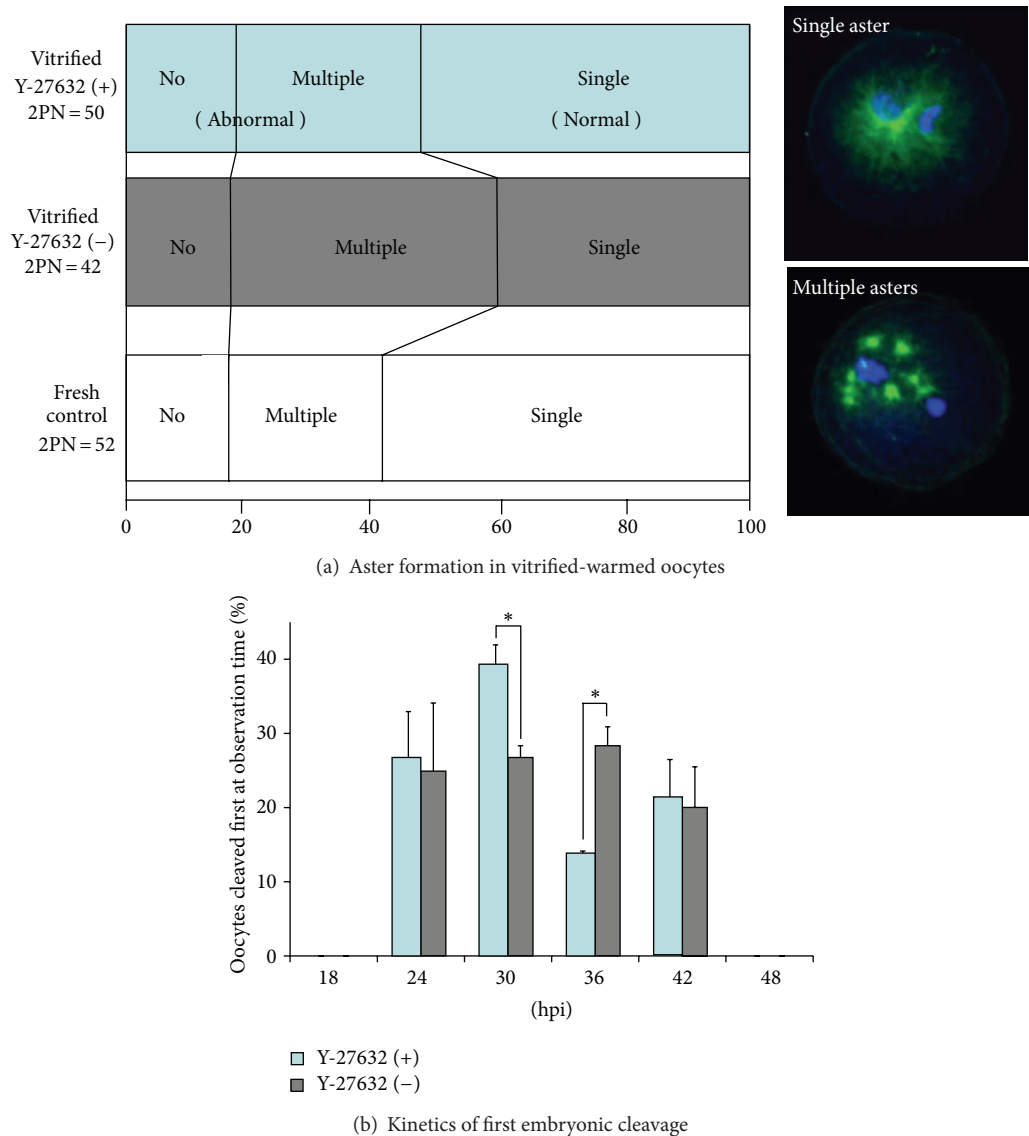


FIGURE 3: Effect of ROCK inhibition during postwarm recovery culture on revivability of bovine mature oocytes [85]. (a) Proportion of vitrified-warmed bovine oocytes exhibiting the formation of no, single, or multiple sperm aster(s). The abnormal incidence of multiple aster formation was inhibited by the recovery culture with Y-27632. Immunostaining against α -tubulin (green) and nuclear staining with DAPI (blue) were performed at 10-hour post-insemination (hpi). (b) Accelerated timing of first cleavage in bovine oocytes vitrified-warmed and rescued with Y-27632. Asterisks indicate significant difference at *P* < 0.05.

the short-term recovery culture of vitrified-warmed oocytes prior to the subsequent IVF. Supplementation of L-carnitine to the IVM medium of bovine oocytes has been reported to redistribute cytoplasmic lipid droplets and improve the cryotolerance of the oocytes after Cryotop vitrification as the blastocyst yield of 34% (comparable to fresh control) [84]. However, it is still unclear whether the positive effect of L-carnitine is reproducible. Incidence of multiple aster formation, a possible cause for low developmental potential of vitrified-warmed bovine oocytes [35], can be inhibited by a short-term culture of the postwarm oocytes in the presence of ROCK inhibitor, with a blastocyst yield of 21% after the Cryotop vitrification (>10% less than fresh control) [85]. Use of an antioxidant α -tocopherol during the recovery culture also rescued the postwarm bovine oocytes as the maximum blastocyst yield at 37% (>10% less than fresh control). Thus, chemical treatment of bovine oocytes before or after the vitrification protocol made it possible to increase their revivability to 20–40% when evaluated with blastocyst yield. Further improvements of the vitrification procedure, combined with pre- and postvitrification chemical treatment, would overcome the high sensitivity of bovine oocytes to cryopreservation and provide valuable information for biomedical experts working in human infertility clinic.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Quantitative Proteomics Analysis of Altered Protein Expression in the Placental Villous Tissue of Early Pregnancy Loss Using Isobaric Tandem Mass Tags

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Many pregnant women suffer miscarriages during early gestation, but the description of these early pregnancy losses (EPL) can be somewhat confusing because of the complexities of early development. Thus, the identification of proteins with different expression profiles related to early pregnancy loss is essential for understanding the comprehensive pathophysiological mechanism. In this study, we report a gel-free tandem mass tags- (TMT-) labeling based proteomic analysis of five placental villous tissues from patients with early pregnancy loss and five from normal pregnant women. The application of this method resulted in the identification of 3423 proteins and 19647 peptides among the patient group and the matched normal control group. Qualitative and quantitative proteomic analysis revealed 51 proteins to be differentially abundant between the two groups (≥ 1.2 -fold, Student's *t*-test, $P < 0.05$). To obtain an overview of the biological functions of the proteins whose expression levels altered significantly in EPL group, gene ontology analysis was performed. We also investigated the twelve proteins with a difference over 1.5-fold using pathways analysis. Our results demonstrate that the gel-free TMT-based proteomic approach allows the quantification of differences in protein expression levels, which is useful for obtaining molecular insights into early pregnancy loss.

1. Introduction

Many pregnant women suffer miscarriages during early gestation, and early pregnancy loss (EPL) has an adverse effect on the quality of life. The incidence of EPL ranges from 50% to 70% [1]. The epidemiological factors contributing to EPL are chromosomal defects of the conceptus, maternal age, endocrine diseases, anatomical abnormalities of the female genital tract, infections, immune factors, chemical agents, hereditary disorders, trauma, maternal diseases, psychological factors, and other such factors [1, 2]. Cytogenetic evaluation of sporadic spontaneous abortions has shown that 50–70% are chromosomally abnormal [3, 4]. The etiologies of EPLs are complex and some occur for unknown reasons. Therefore, the etiology of EPL remains to

be further explored. The placenta is the organ that transports nutrients, respiratory gases, and wastes between the maternal and fetal systems. In the early embryonic development, the placental barrier facilitates the embryo growth in a low oxygen environment, effectively avoiding radical damage; once embryogenesis is complete, the maternal intervillous circulation becomes fully established, and the intraplacental oxygen concentration rises threefold and syncytiotrophoblastic oxidative damage becomes extensive and likely a major contributory factor to miscarriage [5]. The vascular development of the placenta is important for implantation [6]. It appears likely that apoptosis in the cytotrophoblast might be related to one aspect of the proliferation and degeneration of the trophoblast during early pregnancy [7]. There is speculation that an abnormal placenta leads to early abortion,

but the unclear relationship between the placenta and early abortion remains requiring further elucidation.

Proteins that perform biological functions directly are rich in information that has been extremely valuable for the description of biological processes. The correlation between mRNA/DNA and protein levels is insufficient to predict protein expression levels [8]. Proteomics has many advantages compared with other technical means of analysis. Mass spectrometry (MS) allows the multivariate analysis of complex patterns of new biomarkers without knowledge of their individual identities and without having specific antibodies available [9]. A deeper knowledge of the human proteome could help fill the gap between genomes and phenotypes, transforming the way we develop diagnostics and therapeutics, and thereby enhancing overall biomedical research and future healthcare [10]. Recently, proteomics technologies have become sufficiently advanced enough that it is now realistic to measure clinical material to investigate pregnancy-related illnesses such as preeclampsia, Down syndrome pregnancies, and other such conditions [11–14]. The comparison of the proteome of interest between the healthy and disease state thus provides a fine-grained picture of the regulations involved [15]. In 2006, Liu et al. extracted protein from the placental villous tissue of two groups (six placental villous tissues with early pregnancy loss and six from normal pregnant women). They used the proteins from the placental villous tissue of the different groups as a source and 2D-gel-based proteomics as a discovery tool with the aim of discovering differentially expressed externalized proteins in EPL samples [16]. In their study, they identified several proteins associated with placenta-tion and early development, granting new insight into the proteins involved in the pathophysiological mechanisms of early pregnancy loss. 2DE has been a mature technique for more than 25 years and was the first technique capable of supporting the concurrent quantitative analysis of large numbers of gene products. However, in many studies using this technology, the same proteins have largely been identified repeatedly, irrespective of the system studied, which suggests the limited dynamic range of 2DE-based proteomics [17, 18]. In addition, questions remain concerning the ability of the technique to characterize all of the elements of a proteome. Some studies indeed have revealed that typically only the most abundant proteins can be observed using the method [19, 20].

In this study, we aimed to (1) characterize the proteomes of placenta villous tissue using a gel-free proteomics approach, (2) apply high performance liquid chromatography (HPLC)-MS using an isobaric tandem mass tags (TMT) technology to identify differentially abundant proteins between the two groups, (3) bioinformatically analyze the differentially abundant proteins to determine the molecular function, biological process, and signaling pathways using gene ontology (GO) analysis and Pathway Studio software (v6.00), and (4) validate our results by western blot, immunohistochemistry, and pathway analysis of these differentially expressed proteins, which are useful for providing molecular insights into EPL.

2. Materials and Methods

2.1. Clinical Specimen Collection and Preparation. Tissue samples of five early pregnancy loss patients and five normal pregnant women were collected after obtaining informed consent and approval by the Human Studies Committee of Nanjing Maternity and Child Health Hospital (Nanjing, China). The pregnant women providing the samples had vaginal bleeding and/or lower abdominal pain for the first time in the previous few days (0–2 days). The diagnosis of EPL was based on the clinical history, clinical examination, and transvaginal ultrasound (TVU) results. In cases where pregnancy structures (a gestational sac without fetal heart rate) were identified by TVU, the final diagnosis of EPL was made. In control group, pregnancy structures are normal. Inclusion criteria were a gestational age at 7 to 8 weeks (based on the first day of the last menstrual period) and no history of recurrent spontaneous abortions, chromosomal abnormalities, endocrine diseases, anatomical abnormalities of genital tract, infections, immunologic diseases, trauma, internal diseases, hereditary disorders, maternal diseases, psychological factors, or any chemical agent intake before their elective terminations [16]. Experimental and control groups have similar physiological signs.

Placental villous tissue samples were acquired through the cervix during dilatation and aspiration according to strict clinical procedures. The tissue samples were washed in cold normal sterile phosphate buffered saline (PBS) to eliminate maternal blood, and deciduas were removed carefully under a microscope. In addition, each specimen was analyzed by hematoxylin-eosin staining to make sure there is no decidual contamination. Each sample was divided into three parts. One part was used as a cell culture for karyotype assays. Another part was fixed in 4% paraformaldehyde for 24 h at 4°C followed by paraffin embedding, and the third part was stored in liquid nitrogen until extraction. After evaluation via karyotype assays, five normal and five placental villous tissue early pregnancy loss samples, determined not to have embryonic chromosomal abnormalities, were selected for further study and defined as control (CON) samples and EPL samples, respectively.

2.2. Proteomics

2.2.1. Protein Extraction and Digestion. A total of 300 µg of each placental villous sample was homogenized and lysed using a protein extraction buffer consisting of 7 M urea, 2 M Thiourea, 65 mM DTT, and a 1% (v/v) protease inhibitor cocktail. The protein concentration was estimated by Bradford's method [21] using bovine serum albumin as the standard. Cysteine residues were reduced by incubating with DTT for 1 hour at 56°C followed by alkylation with IAA for 45 min at room temperature (RT) in the dark. The protein lysates were cleaned up by acetone precipitation and digested overnight at 37°C with trypsin in a 1:40 enzyme:protein ratio.

2.2.2. TMT Labeling. The six-plex TMTs Label Reagents (Frankfurt am Main, Germany) were equilibrated to room

temperature, and each aliquot was resuspended in 41 μL of anhydrous acetonitrile. Samples were divided into two groups and respectively labeled as follows: Group 1: TMT-126, EPL-1; TMT-127, EPL-2; TMT-128, EPL-3; TMT-129, CON-1; TMT-130, CON-2, and TMT-131 labeled reference sample, which were pooled equally from all the ten samples. Group 2: TMT-126, CON-3; TMT-127, CON-4; TMT-128, CON-5; TMT-129, EPL-4; TMT-130, EPL-5 and TMT-131, pooled reference sample. Each experimental sample was compared to a pooled reference sample. After normalization, all samples were combined for comparison (5 abnormal samples versus 5 control samples) [22]. In addition, 42 μL of the TMT Label Reagents was added to the 100 μg of peptides dissolved in 200 mM triethylammonium bicarbonate (TEAB). After 60 min at RT, 8 μL of hydroxylamine 5% (w : v) was added in each tube, and were incubated for 15 min. The aliquots were then combined, and the pooled sample was evaporated under vacuum.

2.2.3. SCX Fractionation. The peptide mixture was resuspended in SCX chromatography Buffer A (10 mM NH_4COOH , 5% ACN, pH 2.7) and loaded onto a cation ion exchange column (1 mm ID \times 10 cm packed with Poros 10 S, DIONEX, Sunnyvale, CA) with the UltiMate 3000 HPLC system at a flow rate of 50 $\mu\text{L}/\text{min}$. The following linear gradient was used: 0% to 56% B (800 mM NH_4COOH , 5% ACN, pH 2.7) over 40 min, 56% to 100% B over 1 min, 100% B over 3 min, 100% to 0% B over 1 min, and 0% B for 20 min before the next run. The effluents were monitored at 214 nm based on the UV-light trace, and the fractions were collected every 2 min.

2.2.4. LC-MS/MS. Twenty fractions were sequentially loaded onto a μ -precolumn cartridge (0.3 \times 5 mm, 5 μm , 100 \AA ; DIONEX, Sunnyvale, CA) at a flow rate of 0.3 $\mu\text{L}/\text{min}$. The trap column effluent was then transferred to a reverse-phase microcapillary column (0.075 \times 150 mm, Acclaim PepMap100 C18 column, 3 μm , 100 \AA ; DIONEX, Sunnyvale, CA). The reverse-phase separation of peptides was performed using the following buffers: 2% ACN, 0.5% acetic acid (buffer A) and 80% ACN, 0.5% acetic acid (buffer B); a 219 min gradient (0% to 4% buffer B for 8 min, 4% to 9% buffer B for 3 min, 9% to 33% buffer B for 170 min, 33% to 50% buffer B for 10 min, 50% to 100% buffer B for 1 min, 100% buffer B for 8 min, 100% to 4% buffer B for 1 min, and 4% buffer B for 18 min) was used.

Peptides were analyzed using an LTQ Orbitrap Velos (ThermoFinnigan, San Jose, CA) by means of a data-dependent Top10-MS2/MS3 method [23]. For each cycle, one full MS scan of mass/charge ratio (m/z) = 350 to 1800 was acquired in the orbitrap at a resolution of 30,000 or 60,000. Each full scan was followed by the selection of the 10 most intense ions for collision-induced dissociation (CID) and MS2 analysis in the linear ion trap for peptide identification. Each full scan was followed by the selection of the 10 most intense ions for collisioninduced dissociation (CID) and MS2 analysis in the linear ion trap for peptide identification and subsequent higher-energy collisional dissociation (HCD) and

MS3 analysis in the Orbitrap for quantification of the TMT reporter ions [24].

2.2.5. Protein Identification and Quantification. The resulting spectra were searched against the human IPI Protein Sequence Database (version: 3.83, 93, 289 sequences) [25] using MaxQuant (version: 1.2.2.5) software [26]. A common contaminants database was also included for quality control. Except for the TMT quantification labels, carbamidomethylation of cysteine was set as a fixed modification, and oxidized methionine was set as a variable modification. The initial mass tolerances for protein identification on the MS and MS/MS peaks were 20 ppm and 0.5 Da, respectively. Two missed cleavages were permitted, and full cleavage by trypsin was used. The false positive rates (FDRs) of the identified proteins and peptides were estimated by MaxQuant using a reverse strategy. A cutoff value of 1% was used for the identification of peptides and proteins. Protein quantification was calculated by combining MaxQuant identification results with a local modified Libra algorithm [27]. Proteins were considered differentially expressed when they displayed significant changes (more than 1.2-fold and Student's *t*-test *P*-value < 0.05) between the EPL and control groups.

2.3. Bioinformatics Analysis. For the convenience of gene annotation, corresponding Entrez gene IDs of the proteins were used for further bioinformatics analysis. To obtain an overview of the biological functions of the proteins whose expression levels altered significantly in EPL group, gene ontology analysis was performed using WebGestalt (Update 2013) [28]. To further explore the significance of the protein expressional alternation in the EPL group, we used Pathway Studio (v6.00) software (Ariadne Genomics, MD, USA) to search the regulated cellular processes of the proteins that displayed an over 1.5-fold difference between the EPL group and control group.

2.4. Validate the Proteomics Analysis. Western blot analysis of GSTM2 (glutathione S-transferase mu 2 (muscle)), BCS1L (BC1 (ubiquinol-cytochrome c reductase) synthesis-like), CUL7 (cullin 7), and immunohistochemistry analysis on proteins (GSTM2, BCS1L, FAM21 (family with sequence similarity 21), and CUL7) validated the proteomics analysis, they were randomly selected. Detailed steps are in Supplementary Material and method available online at <http://dx.doi.org/10.1155/2014/647143>.

3. Results

3.1. Protein Profiles of Placental Villous Tissue. We successfully identified proteins from placenta villous specimens of the EPL and control groups. In total, 3423 proteins were identified (Data S1) with high confidence (one or more unique peptides with an FDR less than 1%). The detailed information of the identified peptides is shown in Supplemental Data 2.

3.2. Identification of Differentially Expressed Proteins in Placental Villous Tissue from EPL Women by TMT Technology.

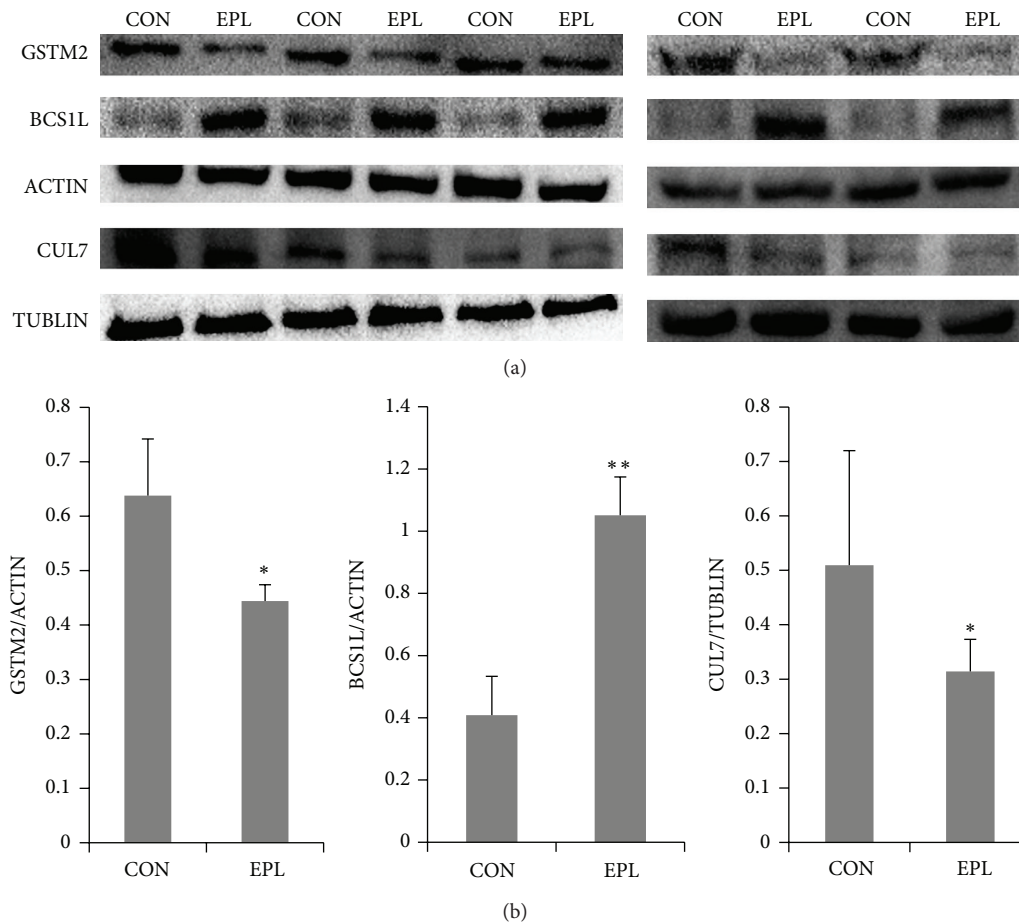


FIGURE 1: (a) Western blot analysis of GSTM2, BCS1L, and CUL7 of the control and EPL samples. (CON), control group; (EPL), EPL group; (b) the bar represents the relative gray values of the western blotting (* $P < 0.05$; ** $P < 0.01$), Student's t -test, P value of GSTM2/ACTIN is 0.013700837; P value of BCS1L/ACTIN is 0.000039685; P value of CUL7/TUBLIN is 0.035421545.

Qualitative and quantitative proteomics analysis revealed 51 proteins as differentially abundant between the two groups (≥ 1.2 -fold, Student's t -test, $P < 0.05$) (Data S3). GO analysis revealed both the molecular function and biological process of the differentially expressed proteins (Graph S1). In terms of molecular function, the most striking tendency is that there are a larger proportion of binding proteins (enzyme binding\ubiquitin protein ligase binding\small conjugating protein ligase binding molecule or a portion thereof). In terms of the biological processes, the top-ranked categories include organonitrogen compound metabolic process and intracellular transport. Among these proteins, 12 proteins were determined to be differentially abundant between the two groups with a fold change ≥ 1.5 (Student's t -test, $P < 0.05$) using this method. Five identified proteins (TBCID13 (TBC1 domain family, member 13), LARP4B (La ribonucleoprotein domain family, member 4B), AHSG (alpha-2-HS-glycoprotein), P4HA2 (prolyl 4-hydroxylase, alpha polypeptide II), and BCS1L) were clearly upregulated, and 7 proteins (GSTM2, CUL7, NES (nestin), RASIP1 (Ras interacting protein 1), SLC30A2 (solute carrier family 30 (zinc transporter),

member 2), PBXIP1 (pre-B-cell leukemia homeobox interacting protein 1), and FAM21) were downregulated in the EPL samples compared with the normal samples.

3.3. Western Blotting Analysis of Differentially Expressed Proteins. To validate proteomics data, we performed western blot analysis on the same lysates of three proteins identified via MS, choosing to analyze proteins with different functions. The proteins confirmed by western blot were GSTM2, BCS1L, and CUL7; they were randomly selected. All of the proteins analyzed by western blot substantially verified the expressional alterations obtained by MS (Figure 1).

3.4. Detection of Differentially Expressed Proteins by Immunohistochemistry. Immunohistochemical studies were performed to examine the expression of some differentially expressed proteins, including GSTM2, BCS1L, CUL7, and FAM21; they were randomly selected. The results indicated the localization of these proteins. There are visualizable

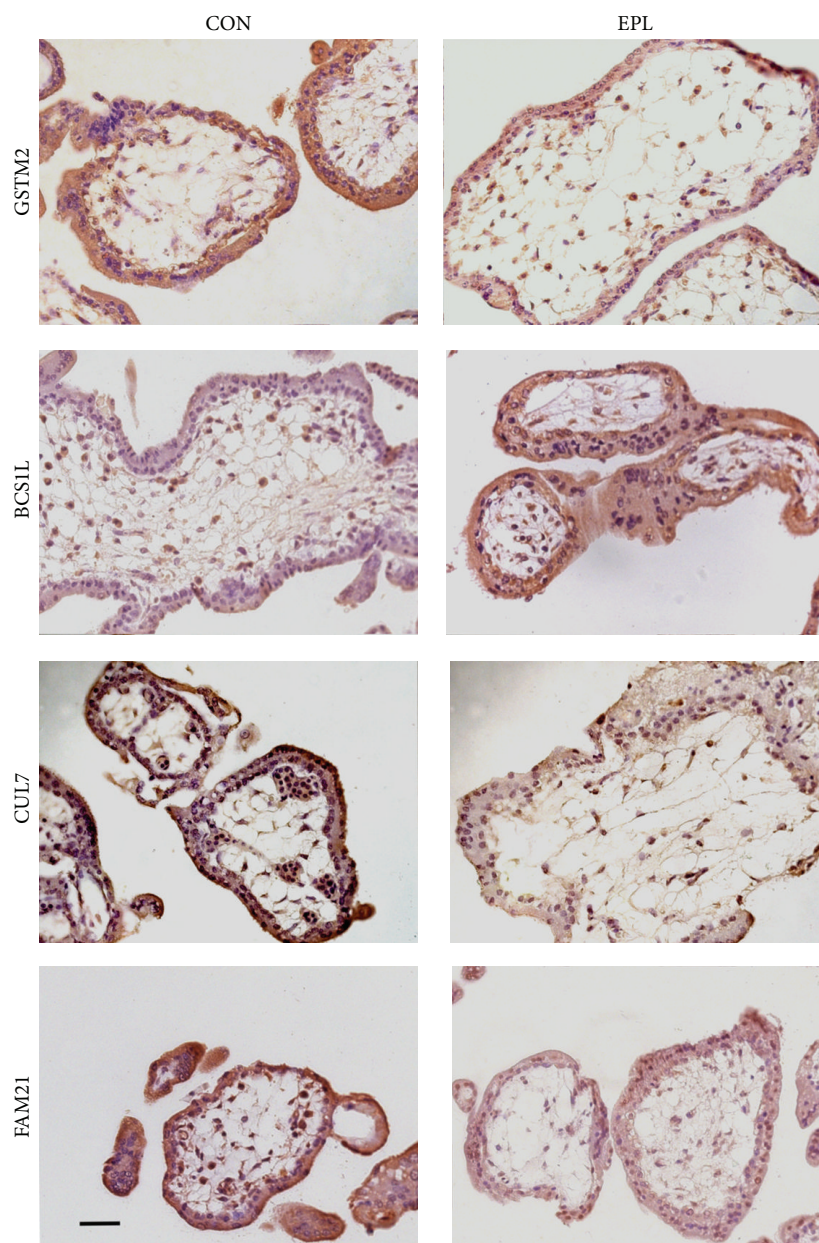


FIGURE 2: Immunohistochemistry analysis of GSTM2, BCSIL, CUL7, and FAM21. In these cases, cytoplasmic staining of the syncytiotrophoblastic and cytotrophoblastic cells was visualized in the placental villous tissues. The trends in the differential expression of the proteins were in accordance with the variation obtained from the quantitative proteomics analysis. (CON), control group; (EPL), EPL group; the bar represents 50 μm .

cytoplasmic staining of syncytiotrophoblastic and cytotrophoblastic cells in the placental villous tissues, and the signals in the tissues of control and EPL group also exhibited a similar expressional tendency as the proteomics data (representative results from each group were shown in Figure 2).

3.5. Pathway Analysis of the Differentially Expressed Proteins.

The simultaneous, cellular process annotation of 12 proteins that were differentially abundant between the two groups (≥ 1.5 -fold, Student's *t*-test, $P < 0.05$) was generated by

Pathway Studio software (v6.00) (Figure 3). The role of these proteins was characterized by cell regulations and processes such as cell migration, angiogenesis, oxidative stress, cell proliferation, apoptosis, and metabolism, among others, which indicates the risk factors in the EPL group.

4. Discussion and Conclusions

The placenta is a major contributor to pregnancy, and many pregnant women suffer miscarriage during early gestation, which has an adverse effect on the quality of life worldwide.

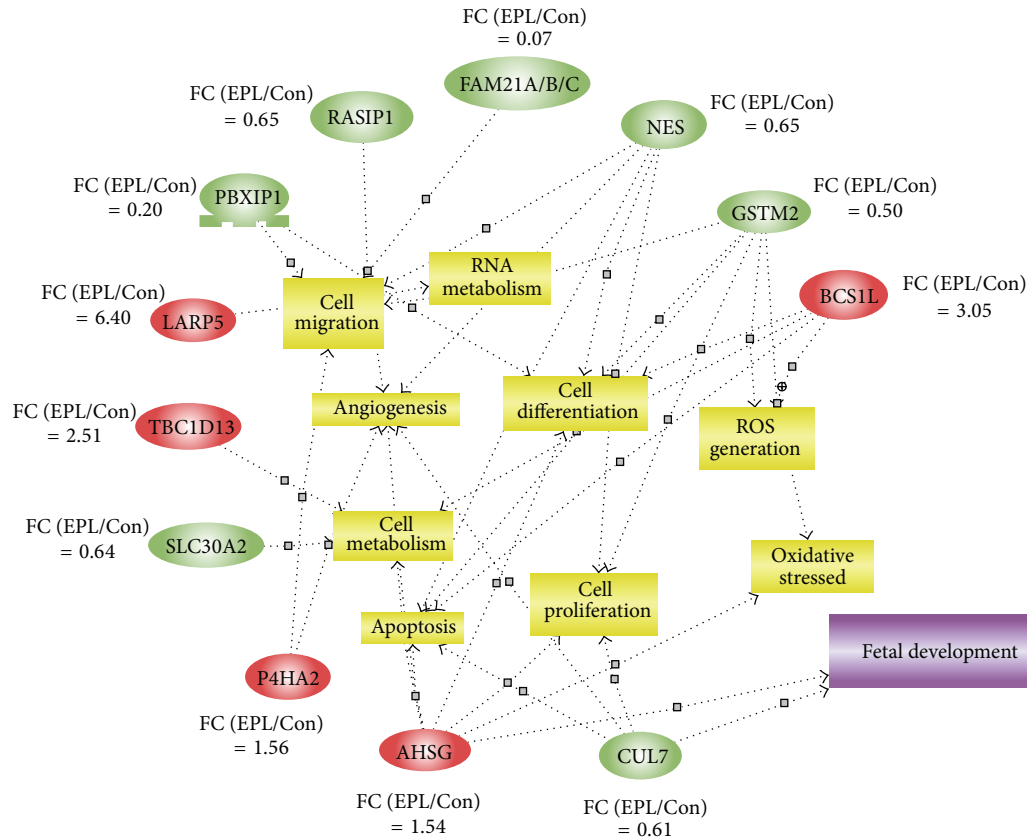


FIGURE 3: The cellular process annotation of 12 proteins that were differentially abundant between the two groups (≥ 1.5 -fold). The cell cycle-related processes were generated by Pathway Studio software (v6.00). Proteins are shown as ovals, (five upregulated proteins were in red and 7 downregulated proteins were in green; the fold-change has been marked on the figures) and the regulated processes are represented by squares. The regulation events are indicated with arrows.

Despite the considerable research efforts expended to understand the causes of these EPL, few significant advances have been made in recent decades because of the complexities of early development. Thus, the identification of proteins with different expression profiles related to EPL is essential to understand the comprehensive pathophysiological mechanism. In this study, we provide unique insights into differentially expressed proteins of placenta villous specimens from EPL subjects via HPLC-MS using TMT-labeling based technology; our data is useful for obtaining molecular insights into early pregnancy loss. We successfully identified 3423 proteins from placenta villous specimens in the EPL and control groups.

Qualitative and quantitative proteomics analysis revealed 51 proteins expressed differentially between the two groups (≥ 1.2 -fold, Student's *t*-test, $P < 0.05$). Using WebGestalt, we performed BP and MF of the differentially abundant proteins. In terms of BP and MF, a majority of the proteins identified were transport/binding/metabolic components. This is because the placenta is an organ of plasticity that adapts to the needs of the fetus during gestation, which is obviously a multistep process [29]. Therefore, gene ontology analysis of differentially expressed proteins involves numerous factors that are involved in these processes, among them, 12 proteins

with a difference over 1.5-fold (Student's *t*-test, $P < 0.05$). The role of these twelve proteins was further characterized by cell regulations and processes such as cell migration, angiogenesis, oxidative stress, cell proliferation, apoptosis, and metabolism among others. Western blot analysis of GSTM2, BCS1L, and CUL7 and immunohistochemistry of the proteins (GSTM2, BCS1L, FAM21, and CUL7) validated the proteomics analysis.

In embryonic development, trophoblast cells adhere and migrate down into the endometrium to form the hemochorial placenta. The early stages of placental development take place in a relatively hypoxic environment that favors cytotrophoblast proliferation rather than differentiation along the invasive pathway [30]. Among the differentially expressed proteins, NES, AHSB, BCS1L, GSTM2, and CUL7 are associated with cell proliferation and apoptosis. Some proteins, such as NES, P4HA2, PBXIP1, and GSTM2, may play a role in cell migration. NES, Cul7, RASIP1, P4HA2, and AHSB are associated with angiogenesis. NES encodes a member of the intermediate filament protein family, which were originally described in neural stem cells [31]. It is consistently expressed in adult angiogenic vasculature. NES expression has also been detected in capillaries of the corpus luteum, which replenishes itself by angiogenesis [32]. In the EPL group of

our study, NES was decreased, which indicates that EPL may have a relationship with angiogenesis. In addition, vascular calcification is the most common type of extra-osseous calcification in end-stage renal disease (ESRD) [33]; AHSG has been demonstrated to exert a calcification inhibitory action both in vitro and in vivo [34, 35]. AHSG is increased in EPL, which may reflect vascular dysfunction in EPL.

The glutathione transferases (GSTs) are a complex family of enzymes involved in detoxification of a wide range of harmful chemicals, including environmental pollutants, carcinogens, mutagens, and toxic products such as lipid hydroperoxides generated during oxidative stress [36]. GSTM2 encodes a glutathione S-transferase. Important clues about oxygen's effects on the placenta have come from several lines of evidence that suggest that the early stages of placental (and embryonic) development take place in an environment that is hypoxic relative to the uterus [30]. As trophoblast invasion of the uterus proceeds, the placental cells encounter increasingly higher oxygen levels [37]. The syncytiotrophoblastic layer of the early placenta is exquisitely sensitive to rapidly rising oxygen tensions in vitro and undergoes selective degeneration [38]. Enzyme manganese superoxide dismutase (MnSOD) catalyzes the dismutation of $O_2^{\cdot-}$ to hydrogen peroxide, which is, in turn, converted to oxygen and water by the enzyme catalase [39]. In our study, GSTM2 expression was low in the EPL groups, suggesting that GSTM2 is important in the maintenance of early pregnancy by preventing the oxidative stress, which is in general agreement with the findings of Liu et al. [16] who reported a decrease in the SOD level in the placental villi of EPL samples.

In addition, the mitochondrial respiratory chain is one of the most prolific producers of superoxide. It has been estimated that almost 1-2% of all electrons passing through the respiratory chain end up as superoxide ions [40]. The production of superoxide during hypoxia and ischemia in cardiovascular tissue can be very damaging [41]. BCSIL encodes a member of the AAA family of ATPases that is necessary for the assembly of complex III in the mitochondria [42]. The AAA-family ATPases mediate the folding, unfolding, assembly, and degradation of proteins [43, 44]. In Björnstad syndrome, BCSIL mutations cause complex III deficiency and GRACILE syndrome, which in neonates are lethal conditions that have multisystem and neurologic manifestations typifying severe mitochondrial disorders [45, 46]. BCSIL mutations alter assembly of the mitochondrial respirasome, reduce the activity of the electron transport chain, and increase the production of reactive oxygen species [42]. BCSIL expression was high in the EPL group, which may reflect the greater production of superoxide and may partly explain the malfunction of GSTM2 in EPL.

CUL7 is a large polypeptide containing a cullin domain, which is present in the anaphase-promoting complex [47]. CUL7 belongs to the cullin family. Cullins are proteins involved in ubiquitination through their participation in multisubunit ubiquitin ligase complexes [48]. Ubiquitination is a critical process in all eukaryotic organisms. It is involved in several essential functions, from the regulation of protein levels to roles in cellular signaling, DNA repair, endocytosis,

and gene expression regulation [49, 50]. Researchers demonstrated that CUL7 is highly expressed in first trimester invasive human placental villi and a key inducer of the epithelial-mesenchymal transition (EMT) of trophoblast lineages [51]. CUL7 appears to be an important regulator of placental development. CUL7 knock-out in mice results in small and abnormal placentas [52]. In this study, CUL7 was decreased in EPL samples compared with normal pregnancy samples. EPL may be associated with ubiquitination and trophoblast migration/invasion. Our studies suggest a possible pathological mechanism for the CUL7-linked pathway in EPL for the first time.

In comparing our results with the published reports, we also observed differences that may be due to the different technical methods, but there are still commonalities identified in the functions of these proteins. Therefore, the use of different methods to elucidate the mechanism of the pathogenesis of EPL is necessary. We conclude that these globally profiled and differentially expressed proteins of placenta villous specimens from EPL women are helpful in obtaining molecular insights into EPL.

Consent

Human Subjects Oversight Approval: tissues were collected after obtaining informed consent and approval by the Human Studies Committee of Nanjing Maternity and Child Health Hospital (Nanjing, China).

Disclosure

Xiaobei Ni, Xin Li, and Yueshuai Guo are similar in author order.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Differential Gene Expression Profiling of Enriched Human Spermatogonia after Short- and Long-Term Culture

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This study aimed to provide a molecular signature for enriched adult human stem/progenitor spermatogonia during short-term (<2 weeks) and long-term culture (up to more than 14 months) in comparison to human testicular fibroblasts and human embryonic stem cells. Human spermatogonia were isolated by CD49f magnetic activated cell sorting and collagen⁻/laminin⁺ matrix binding from primary testis cultures obtained from ten adult men. For transcriptomic analysis, single spermatogonia-like cells were collected based on their morphology and dimensions using a micromanipulation system from the enriched germ cell cultures. Immunocytochemical, RT-PCR and microarray analyses revealed that the analyzed populations of cells were distinct at the molecular level. The germ- and pluripotency-associated genes and genes of differentiation/spermatogenesis pathway were highly expressed in enriched short-term cultured spermatogonia. After long-term culture, a proportion of cells retained and aggravated the “spermatogonial” gene expression profile with the expression of germ and pluripotency-associated genes, while in the majority of long-term cultured cells this molecular profile, typical for the differentiation pathway, was reduced and more genes related to the extracellular matrix production and attachment were expressed. The approach we provide here to study the molecular status of *in vitro* cultured spermatogonia may be important to optimize the culture conditions and to evaluate the germ cell plasticity in the future.

1. Introduction

In humans the process of spermatogenesis is initiated from a small pool of self-renewing stem cells quite late at puberty (10–13 years after birth) and continues throughout life. Human spermatogonial stem cells (hSSCs) have been for the first time identified by Clermont [1]. These cells are positioned in a developmental cascade originating from the embryonic epiblast during gastrulation, followed by primordial germ cells (PGCs) and gonocytes. Although still

a difficult task, the newly established enrichment and *in vitro* propagation of spermatogonia that carry the male genome from generation to generation provide an important step not only for germ cell biology, but also for future transplantation and restoration of fertility in the clinic [2]. Recently, Sadri-Ardekani et al. [3] provided evidence for a potential clinical application by the *in vitro* propagation of prepubertal and adult hSSCs. Furthermore understanding the molecular mechanisms of hSSCs in relation to germ cell cancer development is of massive clinical importance [4].

The strategy of the isolation and short-term cultivation of spermatogonia is in our hands a prerequisite for the generation of pluripotency of these unipotent adult stem cells *in vitro* [5]. The separation of human spermatogonial stem/progenitor cells has been achieved by our group with magnetic activated cell sorting (MACS), using the antibody to CD49f (integrin α -6) followed by matrix selection (collagen nonbinding, laminin binding) to enrich the SSCs from human testis. Several groups successfully established in parallel similar techniques and improved approaches to enrich and culture spermatogonia even for longer time periods [6–11].

Since it is now possible to isolate and culture spermatogonia, there is major interest to understand the self-renewal and germ-associated networks of human adult SSCs and to improve the culture conditions in terms of their stemness and plasticity. It is of utmost importance to show the germ origin of these human testis-derived stem cells that spontaneously behave like pluripotent ESC-like cells that can differentiate into a number of cell lineages comprising the three embryonic germ layers [5, 9, 12–14]. In spite of different approaches in most studies only spermatogonia-enriched cell populations and consequently heterogeneous cell cultures were retrieved, which might mimic the real character and molecular status of spermatogonia during culture *in vitro*. In the studies by Mizrak et al., Chikhovskaya et al., and Gonzalez et al., the cells expressing markers of pluripotency were more mesenchymal stem cell (MSC)-like or probably derived from MSCs [14–16]. In contrast Stimpfel et al. [17] could show that both germ- and mesenchyme-derived cells were present in stem cell clusters from human testis biopsies, which could differentiate in all three germ layers *in vitro* and Lim et al. [18] demonstrated *in vitro* culture-induced pluripotency of hSSCs including teratoma formation. Furthermore renal [19] and hepatic differentiation of hSSCs [20] was observed.

One main step in analyzing the biology of SSCs is to determine their germ cell-specific gene expression profile. The present knowledge regarding the molecular markers that define hSSCs is still significantly limited [21]. The rarity of human testicular tissue available for research, the relatively low number of adult stem cells in the testis, the heterogeneity of human testis tissue available for research, the lack of unique surface markers, and the absence of a robust proliferative *in vitro* culture system to support their self-renewal have prevented so far the efficient isolation and culture of SSCs with high purity for further study. Therefore, the aim of this study was to provide evidence for molecular signatures of human spermatogonia in germ cell cultures, both after short- and long-term culture *in vitro*. In order to accomplish this goal we sought to compare the gene expression profiles of CD49f-positive/matrix enriched human spermatogonia, human testicular fibroblasts (htFibs), and hESCs using microarray analysis and nanofluid real-time PCR (Fluidigm). To get as much a real picture as possible and to avoid the bias because of testicular cell heterogeneity, the cells to be analyzed were individually selected from the spermatogonia-enriched populations cultured *in vitro* by a micromanipulation system.

2. Results

2.1. Selection and Culture of Human Spermatogonia. The spermatogonia were enriched using CD49f-MACS and matrix selection (collagen nonbinding/laminin binding) from orchiectomies (see Tables S1 and S2 (in Supplementary Material available online at <http://dx.doi.org/10.1155/2014/138350>) for patient information related to the SSC cultures). In the selected populations of cells spermatogonia may be the predominating cells, as revealed by positive DDX4 (VASA) and negative VIMENTIN immunocytochemistry of early cultures (Figures 1 and 2), while long-term cultured cells showed reduced VASA staining and were weakly VIMENTIN-positive (Figure 2(b)). The morphology of purified spermatogonia isolated from the different patients was similar irrespective of the age of the patients and days of culture. This was primarily based on their round shape morphology and size of approximately 6–12 μ m in diameter, high nucleus-to-cytoplasm ratio, which could be observed by a clear small shining cytoplasmic ring between the round nucleus and the outer cell membrane. Pairs, chains, and small groups of spermatogonia interconnected by intercellular bridges were present in all cell cultures. In the cultures also some other types of cells, for example, bigger cells with a diameter of 12–14 μ m, an ovoid shape, and a low nucleus/cytoplasm ratio, were observed. htFibs were depleted and mostly remained in the nonselected populations of cells. The htFibs, which easily overgrew the primary cell cultures, were successfully derived from the nonselected cell fractions [18]. Representative examples of spermatogonial cultures devoid of htFibs are shown in Figure 1.

2.2. Pilot Study of Gene Expression Profiling of Enriched Human Spermatogonia after Short-Term and Long-Term Culture with Nanofluidic Real-Time PCR. The nanofluidic real-time PCR gene expression profiling of samples with 50 spermatogonia collected by micromanipulator from short-term culture in one patient (patient 195; duration of culture <2 weeks) and long-term cultures in two patients (patients 184 and 194; duration of cultures between 1 and 6 months) revealed that these cells had a typical germ cell profile (Figure 3 and Figure S1). When analysing all the selected germ-, pluripotency-, and fibroblast-associated genes, profiling showed that short-term cultured spermatogonia separated from all the other groups of cells and were closely related to hESCs in the same dendrogram subtree, while long-term cultured spermatogonia, although positioned in their own subtree, were closer related to htFibs with increasing duration of the culture (Figure 3(a)). This depended on overall changes in the expression of the germ- and pluripotency-associated genes but also related to an increase in more genes related to the extracellular matrix production and attachment during prolonged germ cell cultivation.

The short-term cultured spermatogonia expressed a high level of the known germ cell-specific genes *DDX4* (VASA), *DAZL*, *DPPA3* (*STELLA*), *ZBTB16* (*PLZF*), and the GDNF-receptor *GFR α -1*, while being negative for *c-KIT* (short-term spermatogonia <2 weeks versus htFibs, Mann-Whitney

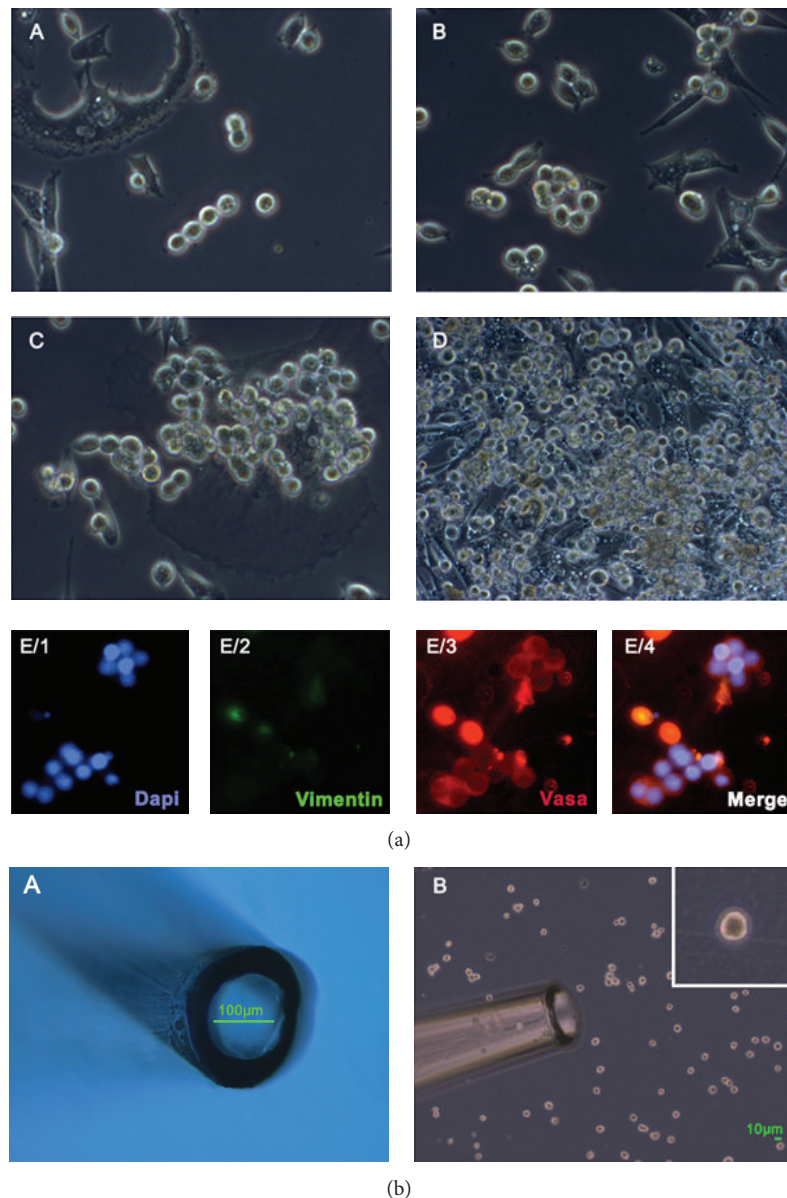


FIGURE 1: Human spermatogonia cultured *in vitro* after CD49f- and matrix-selection. (a) Typical morphology of spermatogonia from same patient 184 during culture: (A) after one week, (B) after one month, (C) after three months, and (D) after six months. Single and interconnected round cells with high nucleus/cytoplasm ratio typical of spermatogonia were observable. Pairs, chains, and small groups/colonies of interconnected spermatogonia were present in all cell cultures. Long-term cultured cells were grown on inactivated CF1 feeder cells. Cultures were devoid of human fibroblasts. (E) Purified VASA-positive germ cell cultures were devoid of VIMENTIN-positive somatic cells. (b) Collection of spermatogonia by a micromanipulation system (micropipette) for gene expression profiling (A). (B) Example of selected spermatogonia is shown as insert. Magnification (a): (A)–(D) 10x, (E/1)–(E/4) 20x; (b): (B) 5x, insert 20x.

test, $P < 0.05$) (Figure 3(b) and Figure S1a). Furthermore these cells highly expressed the pluripotency genes *LIN28*, *NANOG*, *TDGF1*, *CDH1*, *TERT*, and *POU5F1* (*OCT4A*).

In sharp contrast, the htFibs did not express 15 out of the 32 germ- and pluripotency-associated genes, namely, *DDX4* (*VASA*) *DAZL*, *DPPA3* (*STELLA*), *GFRA1*, *PLZF*, *LIN28*, *GDF3*, *NANOG*, *UTF1*, *TDGF1*, *DNMT3B*, *LIN28B*, *TERT*, *CDH1*, and *SOX2*, at all and were positive for *c-KIT*.

Long-term cultured spermatogonia showed reduced expression of *DDX4* (*VASA*), *DAZL*, and *ZBTB16* (*PLZF*) but

stayed positive for the germ cell markers *DPPA3* (*STELLA*) and *GFR α -1*, which were not expressed in fibroblasts (long-term spermatogonia versus htFibs, Mann-Whitney test, $P < 0.05$). Furthermore, the germ and pluripotency cell markers *NANOG*, *LIN28*, *LIN28B*, and *TDGF1* also showed this all-or-none expression pattern as seen in the heat-map for short- and long-term cultured spermatogonia but were not expressed in htFibs (short- and long-term spermatogonia versus htFibs, Mann-Whitney test, $P < 0.05$) (Figure 3(b) and Figure S1a). In comparison to htFibs long-term cultured

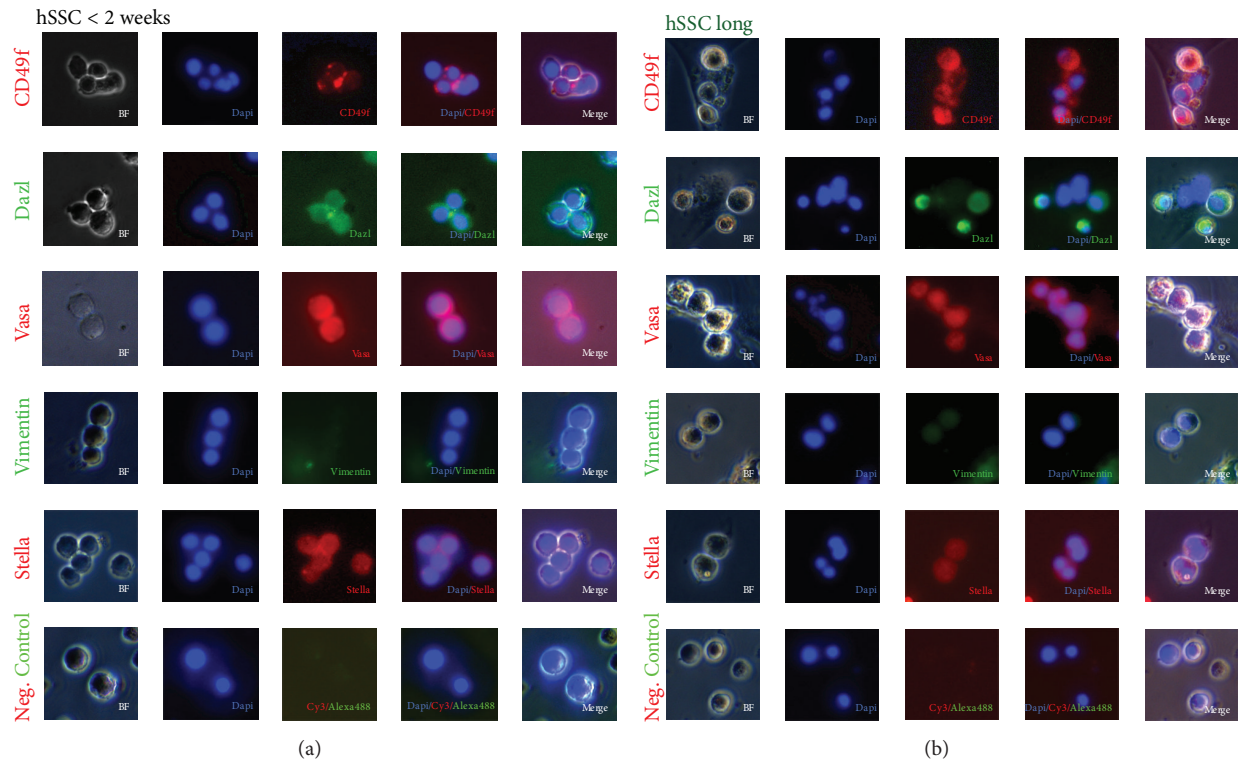


FIGURE 2: Immunohistochemical analysis of (a) hSSC < 2 weeks. Cells were stained with CD49f, DAZL, VASA, VIMENTIN, and STELLA. For VIMENTIN the cultures were negative. Magnification 20x. Immunohistochemical analysis of (b) hSSC long-term cultures. Cells were stained with CD49f, DAZL, VASA, VIMENTIN, and STELLA. For VIMENTIN the cultures were weakly positive. Magnification 20x.

spermatogonia also significantly expressed, although to a lower extent, the germ cell- and pluripotency-related genes *CD9*, *STAT3*, *TDGF1*, *TSPY1*, *OCT4A*, and *NANOS*, while htFibs significantly overexpressed the genes *KIT* and *MYC* in comparison to long-term cultured spermatogonia (long-term spermatogonia <2 weeks versus htFibs, Mann-Whitney test, $P < 0.05$). The pluripotency associated genes *SOX2*, *DNMT3B*, *OCT4A*, *LIN28*, *KLF4*, *STAT3*, and *MYC* were expressed in all long-term cultures of spermatogonia.

The genes *COL1A1*, *FIBRONECTIN1*, and *VIMENTIN*, which were weakly expressed in short-term cultured spermatogonia, were upregulated in long-term cultured spermatogonia (long-term versus short-term spermatogonia <2 weeks, Mann-Whitney test, $P < 0.05$) (Figure 3(b) and Figure S1a). This indicates that long-term cultured spermatogonia expressed more extracellular matrix molecules during prolonged culture, while retaining a germ and pluripotency profile, although the expression of these genes decreased during culture up to 6 months.

As can be seen in the PCAs in Figure S1a, there was a clear difference in the expression of germ and pluripotency genes in short- and long-term cultured spermatogonia and htFibs, although, in the case of long-term cultured spermatogonia, the number of differentially expressed germ cell-associated genes was lower. On the other hand, it became obvious that a part of the genes *COL1A1*, *FIBRONECTIN1*, and

VIMENTIN shifted over to the side of the long-term cultured spermatogonia (Figure S1b).

The real-time PCR data was further supported by immunocytochemical analysis (Figures 2(a) and 2(b)). Short-term cultured spermatogonia stained positive for CD49f, VASA, DAZL, and STELLA and were negative for VIMENTIN. Long term-cultured cells showed weaker signals for CD49f, VASA, DAZL, and STELLA and stained slightly positive for VIMENTIN.

These observations encouraged us to have a closer look at the changes in genes' global expression and in particular the expression of germ-, pluripotency-, and fibroblast-associated genes and genes activated during the short- and long-term culturing of human spermatogonia.

2.3. Microarray Gene Expression Profiling of Human Spermatogonia

2.3.1. Dendrograms of Sample Relations. The dendrograms of sample relations display the distances or degree of relationship between experimental samples (Figure 4). Three different dendrograms were calculated, comparing germline-derived cells: short- and long-term cultured hSSCs to somatic cells: htFibs and hESCs. All dendrograms were created using hierarchical complete linkage clustering with Euclidean

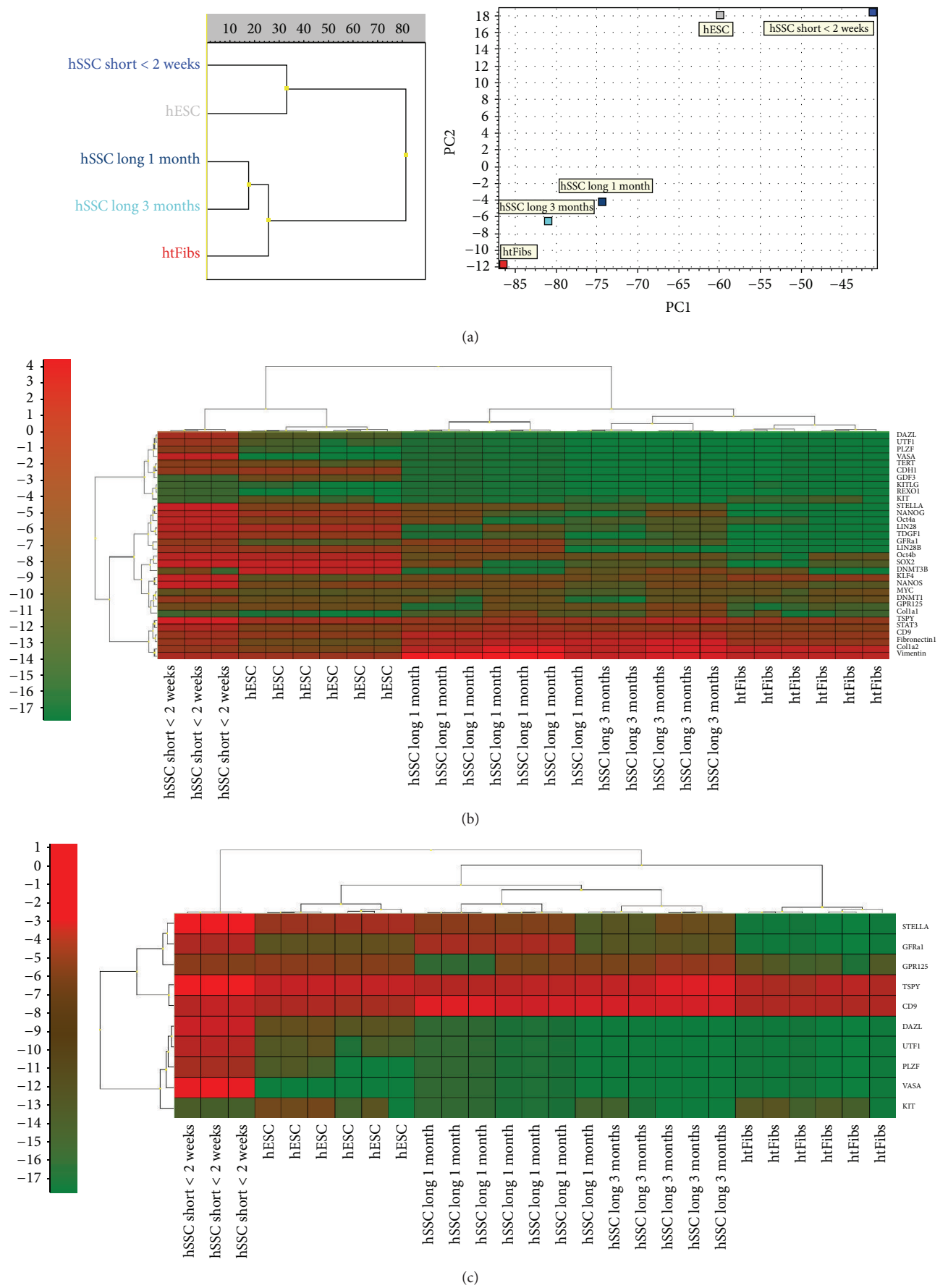


FIGURE 3: Continued.

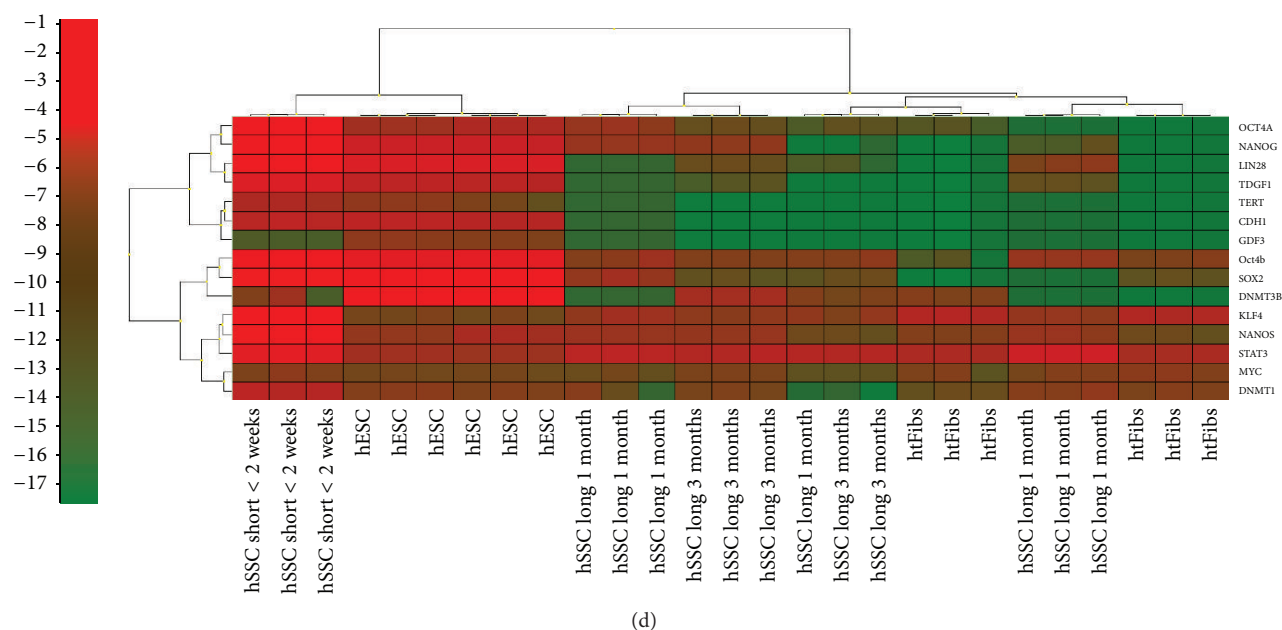


FIGURE 3: Germ-, pluripotency-, and fibroblast-related gene expression profiling of samples with 50 spermatogonia from short-term (patient 195) and long-term cultures (patients 184, 194) in comparison with human embryonic stem cells and human testis fibroblasts with Fluidigm BioMark system. (a) Dendrogram and PCA. Heatmaps ranging from strongly expressed (red) to absent (green) with hierarchical clustering are shown (b) for all analysed genes, (c) for germ- and (d) for pluripotency-associated genes.

distance. The three dendrograms were calculated: (1) using the whole transcriptome assessed by the Affymetrix platform (Figure 4(a)), (2) considering only 150 features with highest variances (Figure 4(b)), and (3) using the 150 features with highest discrimination power assessed by the P value of an F -test (Figure 4(c)).

On the level of whole transcriptome, hSSC short- and long-term cultures cells were clearly separated into different subtrees (Figure 4(a)). In particular short-term cultured spermatogonia were substantially different from the other cell types. hESCs had their highest degree of relationship to fibroblasts being located in the same subtree. One of the htFibs samples seems to be more closely related to hESCs. Looking at the dendrogram, calculated from the 150 high variance features (Figure 4(b)), the separation of the cell types became even more evident and all cell types were grouped correctly into subtrees. Again, the hSSC short-term cultured cells were very distinct from the other cell types. The previously outlying htFibs sample was now similar to the other htFibs. The separation of the cell types was even stronger using the top 150 features with most significant P values of an F -test (Figure 4(c)).

This indicates that short-term cultured spermatogonia from one patient join at similar levels, as do long-term cultured spermatogonia from two patients, and also htFibs and hESCs. All cell types have their distinct expression pattern and they are clearly distinguishable from each other. This was true for the selected set of genes based on high variance genes but also visible using the complete transcriptome (Figure 4).

2.3.2. Differential Analysis of Short- and Long-Term Cultured Spermatogonia in Comparison with Somatic htFibs and hESCs. Differential analysis identified genes up- or downregulated in the two sets of samples. Standard t -test comparing groups were performed. Figures 5 and S2 show volcano plots visualizing fold changes and t -test P values of the comparisons of the short-term and long-term cultured spermatogonia with the control group composed of htFibs and hESCs (Figure 5). For both analyses genes showed strong fold changes up to a 32-fold differential regulation (\log_2 folds up to 5).

Differential analyses were used to define lists of germ cell-associated genes upregulated in short- (SSC.short: *PROTAMIN 1*, *PROTAMIN 2*, *TNP1*, *CRISP2*, *ODF1*, *ODF2*, *ADADI*, *PHF7*, *SPATA6*, *TMEM31*, *GSF1*, *MAEL*, *C9ORF9*, and *GTSF1*) and long-term cultured spermatogonia (SSC.long: *PTN*, *MARCKS*, *SMAD5*, *POSTN*, *ALDHIA2*, *SRFP1*, *CUL3*, *HS6ST2*, *PTEN*, *LIFR*, *CCND2*, *CXADR*, and *RGS4*) when compared to testis htFibs and hESCs.

Additional sets of literature-derived pluripotency-associated genes (pluripotency: *POU5F1* (*OCT4*), *LEFTY2*, *CDX2*, *FOXO1*, *LIN28A*, *HAND1*, *DPPA4*, *SOX2*, *ZIC3*, *GJAI*, and *NANOG*) and germ cell-enriched genes (GS genes: *TSPYL1*, *DDX4* (*VASA*), *UTF1*, *CD9*, *ZBTB16* (*PLZF*), *TSPYL1*, *GPR125*, *DAZL*, *GFR α -1*, *DPPA3* (*STELLA*), *NANOS2*, *NANOS1*, *NANOS3*, *KIT*, and *LIFR*) were defined. Genes in these lists are highlighted in the volcano plots.

Next we focused on these gene sets and visualized gene expression in a heat map (Figure S3). The corresponding volcano plots are provided as supplemental material (Figure

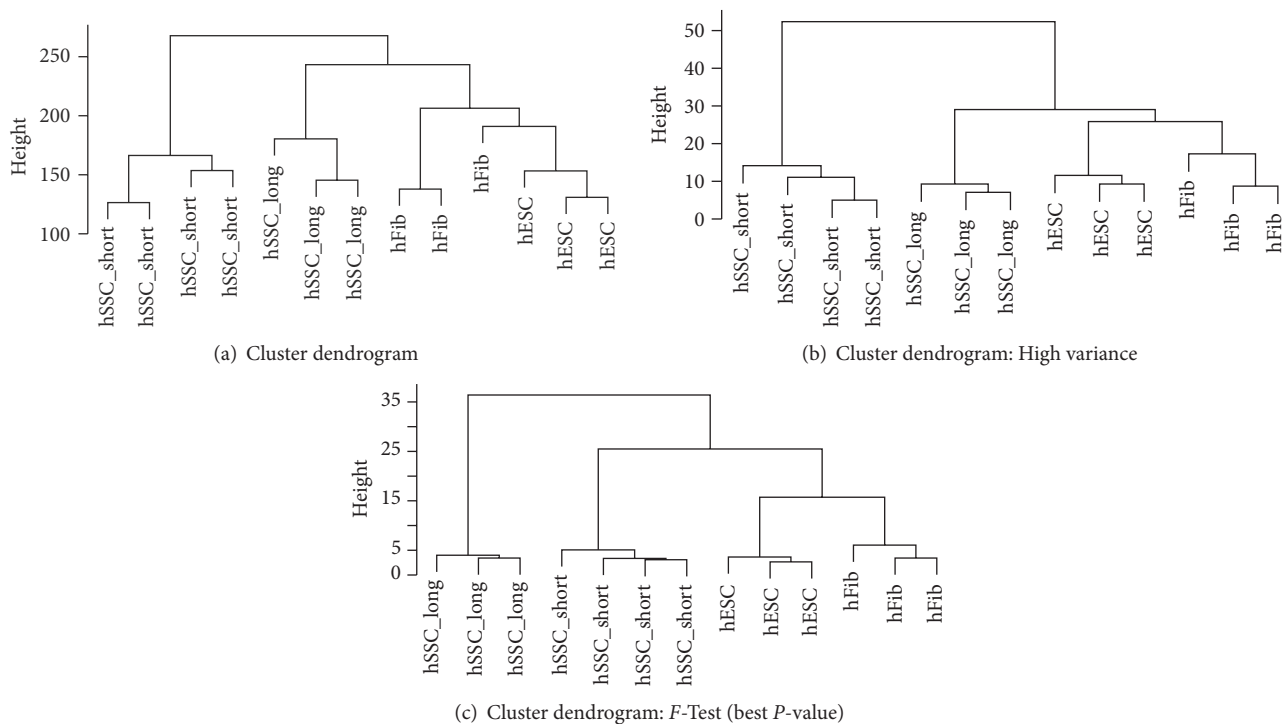


FIGURE 4: Genome-wide profiling of spermatogonia in comparison with htFibs and hESCs. (a) Dendrogram based on the whole transcriptome, calculated using complete linkage clustering and Euclidean distance. Samples were grouped almost perfectly according to their cell type. Short-term cultured spermatogonia (spermatogonia_short, left hand side of the tree) were poorly related to other cell types. hESCs have a rather high degree of relationship to fibroblasts. (b) Dendrogram based on the 150 features with highest variances, calculated using complete linkage clustering and Euclidean distance. All cell types were grouped correctly into subtrees. (c) Dendrogram based on the 150 features with highest discrimination power assessed by the P value of an F -test. Using a set of markers designed for distinguishing the groups, separation of the cell types was even stronger in comparison with (a) and (b). The overall topology remains very similar in all cases.

S2), with tables showing the 20 most strongly expressed genes and the 20 most upregulated stem (pluripotency) and germ-cell-associated genes (Table S3).

2.4. Control Cells. When comparing control htFibs and hESCs based on the selected gene lists it became clear that, as expected, pluripotency-associated genes *POU5F1* (*OCT4*), *SOX2*, *NANOG*, *LEFTY2* and *DPPA4*, *GJA1*, and *LIN28A* were higher expressed in hESCs (log2 fold up to 5.2), as seen in Figure S3A. Interestingly, also some of the germ cell-associated genes: *SRFP1*, *PTN*, *CXADR*, *CCND2*, *ALDH1A2* (from SSC_long list), *SPATA6*, *PROTAMINI* (*PRMI*), *TNPI*, *ODF1*, and *ODF2* (from SSC_short list) were higher expressed in hESCs compared to htFibs. In the comparison of htFibs and hESCs, among the 25 genes listed above for short- and long-term cultured spermatogonia (SSC_short and SSC_long genes) only *POSTN* is differentially overexpressed in htFibs by more than log2 fold >2.

2.4.1. Short-Term Cultured Spermatogonia. When comparing short-term cultured spermatogonia with htFibs and hESCs, the established germ cell markers *DDX4* (*VASA*), *TSPY1*, and *DAZL* (GS genes), which were mostly expressed in germ stem and progenitor cells, were upregulated in spermatogonia (log2 folds up to 3.3), as can be seen in Figure 5 and Figures

S3 and S4. In addition, *TSPY3* was also strongly upregulated. Other known germ cell markers were not differentially expressed. ES-specific genes *POU5F1*, *NANOG*, *LIN28*, and *LEFTY2* showed high expression in hESCs but low expression in short-term cultured spermatogonia. The most striking observation was that many upregulated genes (with log2 folds up to 5.5) were known to be related to germ cells. The most upregulated genes were associated with the germ cell differentiation pathway and include genes important for spermatogenesis, such as *PROTAMIN2* and *PROTAMINI* (*PRM2*, *PRM1*), *TMEM31*, *CRISP2*, *PHF7*, *ODF2*, *ADAD1*, *TNPI*, and *SPATA6* (see also volcano plot in Figure 4). The germ cell-associated genes *CUL3*, *ALDH1A2*, and *PTEN* from SSC_long gene list were also upregulated in the short-term cultured cells as well (log2 folds up to 3.3). It has to be pointed out that most of the germ cell-associated genes (GSC genes) and those highly upregulated in short-term cultured spermatogonia (SSC_short genes) had low expression in htFibs and hESCs. It also should be noted that several genes, classified as typical spermatogonial markers, such as *ZBTB16* (*PLZF*), *GFR α -1*, and *DPPA3* (*STELLA*) were not found to be highly expressed on the Affymetrix chips.

2.4.2. Long-Term Cultured Spermatogonia. In comparison of long-term cultured spermatogonia with htFibs and hESCs, no

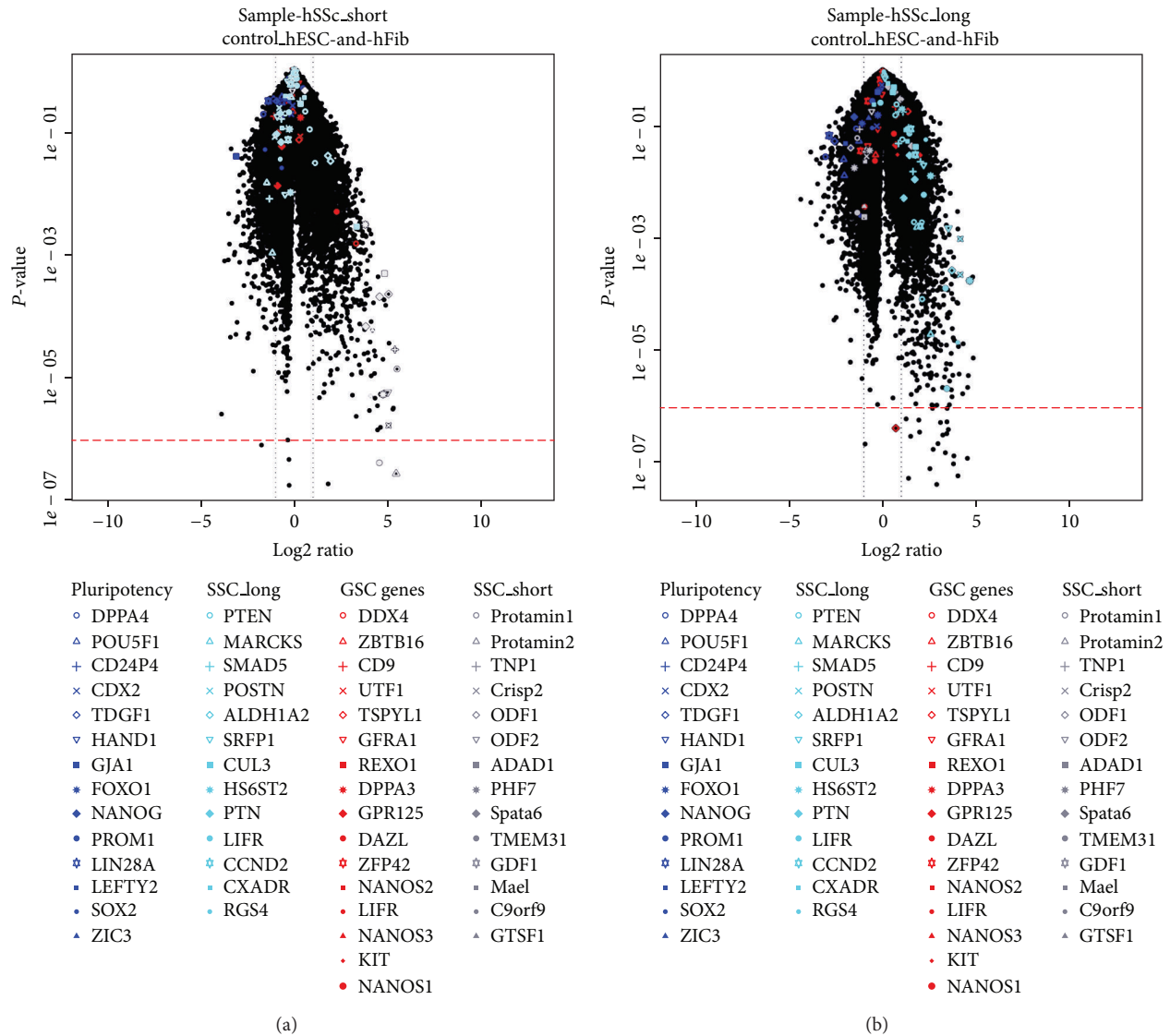


FIGURE 5: Volcano plots (x-axis: log2 ratio and y-axis: *t*-test *P* value). (a) SSC_short (short-term cultured spermatogonia) versus hESCs and htFibs. (b) SSC_long (long-term cultured spermatogonia) versus hESCs and htFibs. Expression of four different sets of genes (pluripotency-, GS genes, SSC_short-, and SSC_long-associated genes) were analysed.

pluripotency-associated genes were upregulated in these cells (Figure 5 and Figures S3 and S4). However, *GPR125*, *LIFR*, *KIT*, and *KITLG* from the known germ cell markers (GSC genes) showed the highest expression in long-term cultured spermatogonia. Only a single probe for *SPATA6* gene from the list of short-term cultured spermatogonia (SSC_short genes) was upregulated with a log2 fold of 1.5. A number of the highly expressed markers in long-term cultured SSCs (SSC_long genes) were stem- and germ cell-related genes. The most upregulated genes in this group were *HS6ST2*, *POSTN*, *SFRP1*, *ALDH1A2*, *LIFR*, *CCND2*, *RGS4*, *MARCKS*, *PTEN*, and *PTN*. Several genes from the SSC_long list were highly

expressed also in hESCs (*MARCKS*, *PTEN*, *CCND2*, *PTN*, *PTEN*, *SFRP1*, and *CUL3*) but not in fibroblasts.

2.4.3. Differences between Long-Term and Short-Term Cultured Spermatogonia. When comparing short- and long-term cultured spermatogonia directly with each other, short-term cultured spermatogonia showed an upregulation of the pluripotency-associated genes *POU5F1* (*OCT4*), *LEFTY2*, and *DPPA4* (log2 folds 1.5) and of the germ cell-associated genes *DDX4* (*VASA*) (log2 fold 4.25), *TSPY1* (log2 fold 3.8), and *DAZL* (2.66) (Figure S2). Most of the short-term

TABLE 1: Short-term cultured spermatogonia were significantly represented in a statistical evaluation of this unbalance.

WordStat-sample-hSSC.short versus control-hESC and -htFib and -hSSC.long				
	Number	Mean difference	<i>t</i> -test	Wilcoxon <i>P</i> value
Sperm	162	0.6219	$3.334e - 10$	$1.208e - 15$
Testis	72	0.9562	$6.729e - 10$	$4.69e - 19$
Meiosis	10	0.3853	0.04165	0.01713
Germ	8	0.4991	0.1311	0.02802
Gamet	7	1.774	0.03857	0.001336

SSC-associated genes (SSC_short genes) showed rather low expression in long-term cultured spermatogonia. Some genes from the SSC_long gene list also showed high expression in short-term cultured spermatogonia (i.e., *PTEN* and *CUL3*).

2.4.4. Analysis of Short- and Long-Term Cultured Spermatogonia with Predefined Gene Sets for Germ-, Pluripotency-, and Fibroblast-Associated Genes from the Literature. In an extended approach, we now considered different predefined sets of genes: fibroblast specific genes, hESC-enriched genes, and genes found to be enriched in fibroblasts. The genes were extracted from the publication of Ko et al. 2010 [22] (Figure 1k: human ES cell-enriched genes). Expression of the three different gene sets (fibroblast-specific genes, ES-enriched genes, and fibroblast-enriched genes) is presented in heatmaps in Figure S4 and in Figure 6(a) which corresponded to Figure 1k from the publication of Ko et al. [22].

Interestingly, gene *HOOK1* was strongly expressed in short-term cultured spermatogonia. In long-term cultured spermatogonia genes *LRRN1* and *CXADR*, both of which are linked to germ cells, were upregulated.

Short- and even more interestingly long-term cultured spermatogonia expressed several genes from the list of genes involved in extracellular matrix production and attachment (Figure S4). However, especially for the short-term cultured spermatogonia, there were substantial differences relative to htFibs.

A correlation matrix of the four different cell types based on the five gene sets (pluripotency genes, GS genes, fibroblast-specific genes, ES-enriched genes, and fibroblast-enriched genes) is shown in Figure S5. Short-term cultured spermatogonial cells showed similar correlation to hESCs (Pearson correlation coefficient $\rho = 0.51$) and to htFibs ($\rho = 0.54$). Short- and long-term cultured spermatogonia were more distinct ($\rho = 0.43$). In particular long-term cultured spermatogonia were different from hESCs based on this data subset ($\rho = 0.08$), but they were more similar to htFibs ($\rho = 0.72$) using five gene sets.

While htFibs were quite different from hESCs ($\rho = 0.17$), they had similarities to the short-term cultured spermatogonia ($\rho = 0.54$) and long-term cultured spermatogonia ($\rho = 0.72$). Since, according to morphological criteria, no fibroblasts were selected for the analysis from the germ cell cultures, the increase of fibroblast-enriched genes during long-term spermatogonia culturing may be related to culture conditions on feeder layers and growth factors.

2.4.5. Differential Analysis of Microarray Data with Test of Germ Cell-Associated Terms. In a text mining approach we investigated if the germ cell-specific terms: “sperm,” “testis,” “meiosis,” “germ,” and “gamete” were annotated for the genes. To this end gene descriptions, retrieved from BioMart, were parsed for the occurrences of these terms. Features, whose description contain at least one of the terms, were coloured in a volcano plot of hSSC_short and control cells in Figure S5 and Table 1. An unbalanced left/right distribution of a colour indicates a relation of the annotated term with the sample/control grouping. For statistical assessment of this unbalance we tested whether log2 ratios for genes containing a certain term were identical to log2 ratios for genes that do not contain the term based on *t*-test and Wilcoxon’s test. The results are shown in Table 1. Genes annotated with “sperm” or “testis” were significantly upregulated in hSSCs compared to the control group. For long-term spermatogonia this association was not observed.

The differential analysis of the microarray data clearly indicates that the CD49f and matrix selected short-term (<2 weeks) cultured cells were not bulk fibroblasts [18] but rather showed individual germ cell specific expression patterns. This broad variation in expression was due to cell type-specific genes and may serve as a fingerprint for spermatogonia.

2.5. Validation of Microarray Results by Nanofluidic Real-Time PCR. The initial set of markers for characterizing genes enriched in human SSCs included the germ cell-specific genes: *TSPYL*, *DDX4* (*VASA*), *DAZL*, *ZBTB16* (*PLZF*), *DPPA3* (*STELLA*), *CD9*, *NANOS*, *UTF1*, *LIFR*, *KIT*, *KILG*, *REX1*, *GPR125*, and *GFRa1*, and the pluripotency-associated genes: *POU5F1* (*OCT4*) A, *LIN28A*, *NANOG*, *SOX2*, and *GDF3*. In addition the expression of OCT4B was measured. Based on the microarray data analysis of the group of short-term cultured spermatogonia, the following germ cell- and spermatogenesis-associated genes were selected for the real-time PCR analysis: *PRM1*, *PRM2* 2, *TNPI*, *CRISP2*, *ODF1*, *ODF2*, *ADAD1*, *PHF7*, *SPATA6*, *TMEM31*, *MAEL*, *C9ORF9*, and *GTSF1*, as based on high expression levels (see Tables S3A and S3C). For gene expression profiling of long-term cultured spermatogonia the following genes, associated with stem/germ cells and spermatogenesis, were selected: *PTN*, *MARCKS*, *SMAD5*, *POSTN*, *ALDH1A2*, *SRFP1*, *CUL3*, *HS6ST2*, *SOX9*, *COLEC12*, *TSPYL1*, and *RGS4* based on high expression levels (see Tables S3B and C).

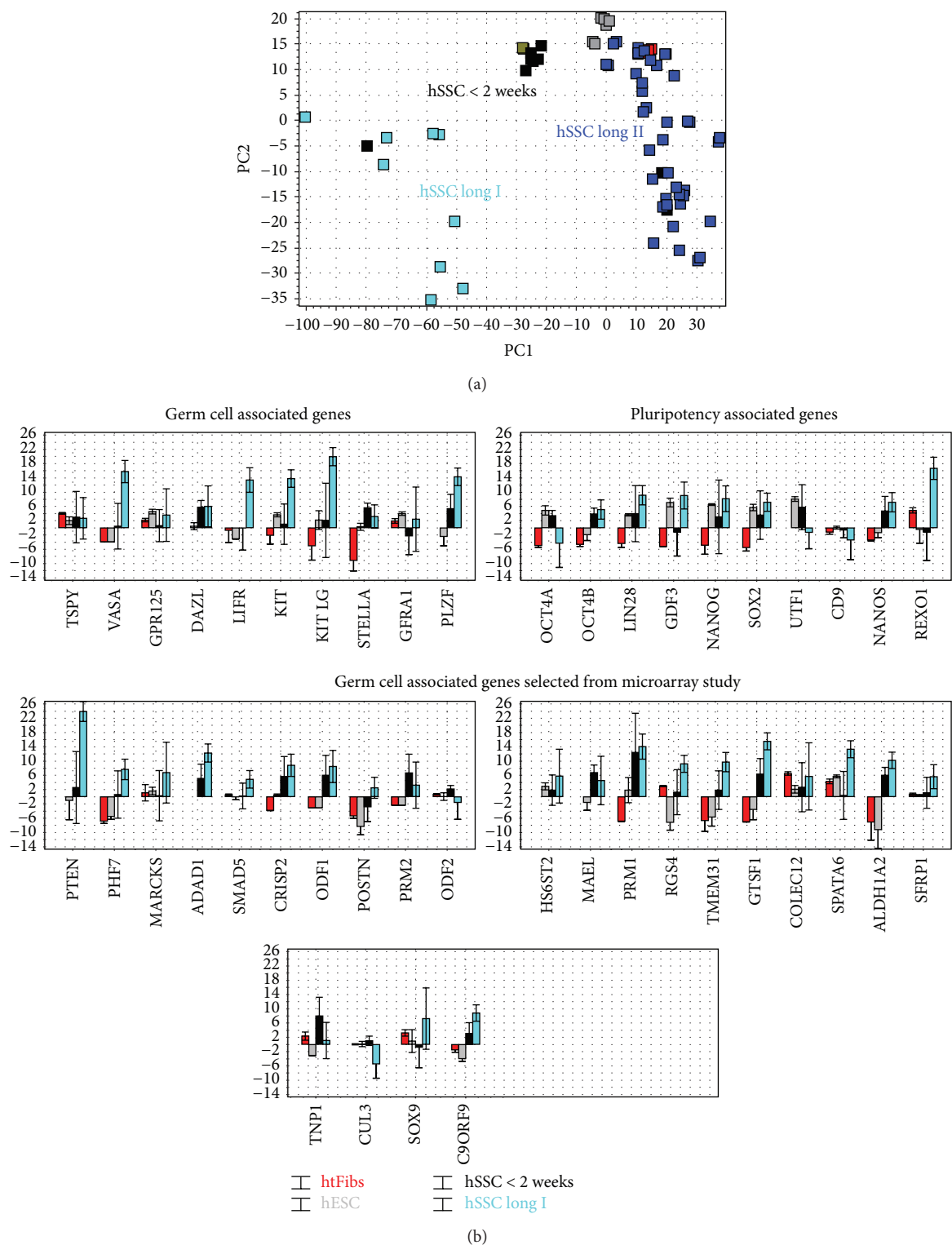


FIGURE 6: Analysis of all long-term cultured spermatogonia by real-time PCR. PCA shows that htFibs, hESCs, short-term cultured hSSCs, and a group of long-term cultured hSSCs (group I) clearly separate. (b) Bar plots of long-term cultured spermatogonia (group I) in comparison to short-term cultured spermatogonia, hESCs, and htFibs: (a) For germ cell-associated genes, (b) pluripotency-associated genes, and (c) germ cell-associated genes selected from microarray study. Coloring: htFibs: red; hESCs: grey; short-term cultured spermatogonia from patient 219: black; long-term cultured spermatogonia from group I: green; long-term cultured spermatogonia from group II: blue.

2.5.1. Human Testis Fibroblasts (htFibs). According to the Fluidigm real-time PCR analysis 14 out of the 45 transcripts were found completely absent in somatic htFibs, including genes *DDX4* (*VASA*), *DAZL*, *UTF1*, *KITLG*, *ADAD1*, *CRISP2*, *ODF1*, *PRM1*, *PRM2*, *MAEL*, *GTSF1*, *PTN*, *HS6ST2*, and *GDF3*. Furthermore, the genes *STELLA* and *GFRA1* showed very low expressions with CTs > 25 cycles in htFibs. These genes are all associated with germ cells but for *GDF3*, which belongs to the group of pluripotency enriched genes and may therefore be used as marker to distinguish human spermatogonia from testis fibroblasts.

2.5.2. Short-Term Cultured Spermatogonia. When the expression of genes from the three subsets measured on the Fluidigm were compared between htFibs, hECSs, and short-term cultured spermatogonia (patient 219) similar results to those from the microarray analysis (patients 189 and 195) were obtained. In hierarchical cluster analysis hECSs and htFibs localize to the same subtree and are clearly separated from the short-term cultured spermatogonia (Figure 6). Also from the PCA (Figure S6) it is evident that short-term cultured spermatogonia cells are different from the other cell types.

Several of the markers characteristic of germ cells: *DAZL*, *STELLA* (*DPPA3*), *PLZF* (*ZBTB16*), *NANOS*, and *UTF1*, and the pluripotency-associated genes *POU5F1A* and *SOX2* were enriched in the short-term cultured hECSs (short-term spermatogonia <2 weeks, patient 219 versus htFibs, Mann-Whitney $P < 0.05$), while genes *TSPYL*, *VASA* (*DDX4*), *PLZF* (*ZBTB16*), *GPRI25*, *LIN28*, *NANOG*, *GFR α -1*, *KIT LG*, *LIFR*, and *GDF3* were not significantly differentially expressed in this patient (short-term spermatogonia <2 weeks, patient 219 versus htFibs, Mann-Whitney $P > 0.05$). When two outliers were removed from the group of cells collected from patient 219, all genes but *LIFR* were now found significantly differentially expressed. The genes *PRM1*, *PRM2*, *TNPI*, *CRISP2*, *ODF1*, *ODF2*, *GTSF1*, and *MAEL* that were overexpressed in short-term cultured spermatogonia in the microarray analysis were also found overexpressed by real-time PCR, and we conclude that expression of these markers clearly distinguishes short-term cultured spermatogonia from hECSs and htFibs (Figure 6). The genes that had the highest expressions (fold change) in the short-term cultured spermatogonia in comparison to htFibs were *PRM1*, followed by *STELLA*, *GTSF1*, *ALDH1A2*, and *PLZF*, and in comparison to the hECSs they were *ALDH1A2*, *TNPI*, and *PRM1*. From the group of long-term enriched genes *GTSF1*, *ALDH1A1*, *MAEL*, *CRISP2*, *TMEM31*, and *ADAD1* were upregulated in short-term cultured spermatogonia compared to htFibs (Mann-Whitney test, $P < 0.05$) (Figure 6).

2.5.3. Long-Term Cultured Spermatogonia and Overall Comparisons of Short- and Long-Term Cultured Spermatogonia. The PCA results in Figure 6 separating htFibs, human ES cells, short-term and long-term cultured spermatogonia were analysed in more detail. The following features were apparent: a compact cluster of “short-term cultured spermatogonia” samples from patient 219 with two outliers (red), a compact

cluster of htFibs (purple), a compact cluster of hECSs (grey), and separation of “long-term cultured spermatogonia” into two major groups (green), a small group of nine samples (group I) from four patients, 184, 191, 201, and 203 (see Tables S1 and S2), and a large group of 40 samples (group II) from six patients, 184, 191, 196, 201, 203, and 214 (Figure 6).

The expression profile of group I consisted of the germ cell genes *VASA*, *PLZF*, *LIFR*, *KITLG*, *KIT*, *STELLA*, *DAZL*, *REXO1*, and *TSPY1* and the pluripotency-associated genes *NANOS*, *LIN28*, *NANOG*, *SOX2*, and *REXO1*, from the differentiation pathway *CRISP2*, *ODF1*, and *PRM1*, and from the group of long-term cultured associated genes *PHF7*, *POSTN*, *TMEM31*, *PTEN*, *ADAD1*, *ALDH1A2*, *SMAD5*, *RGS4*, *SPATA6*, *SFRP1*, *CUL3*, and *C9ORF9* (Mann-Whitney test, $P < 0.05$, compared to htFibs) (Figure 6). In group II only the genes *OCT4A*, *OCT4B*, *PHF7*, and *POSTN* were significantly upregulated relative to htFibs (Mann-Whitney test, $P < 0.05$, compared to htFibs).

In general, the “long-term cultured spermatogonia” samples had similar PC1 scores, suggesting that expression of the genes behind PC1 is fairly homogeneous among those samples, while the genes behind PC2 show variability (Figure S6). Interestingly, the PC1 score for the “long-term cultured spermatogonia” samples is similar to that of the htFibs and of the hECSs, suggesting that they have common expression of the underlying genes. In this analysis all plots reveal four “long-term cultured spermatogonia” and two “short-term cultured spermatogonia” outlier samples characterized by a very low PC1 score. When including more genes in the classification the separation of htFibs and “long-term cultured spermatogonia” disappears in the PC1 versus PC2 plot. However, it remains in the 3D plot that reflects also the contribution from PC3, evidencing different expression profiles (Figure S6).

Notable, the “short-term cultured spermatogonia” samples are in the centre, while the “long-term cultured spermatogonia” samples are located at the periphery of the PC1 versus PC2 plot. The separation is even more evident in a 3D PC1 versus PC2 versus PC3 plot. This evidences samples changing expression profiles as the spermatogonia ages in the culture, and the changes are not homogeneous. Rather, the samples differentiate into three different directions reflected by moving towards high PC1 or high or low PC2. The genes important for the separation along the three axes include *ALDH1A2*, *PHF7*, *NANOG*, *LIN28*, *SOX2*, *MARKS*, and *POSTN*.

The expression profiles of germ cell-enriched genes in short- and long-term cultured spermatogonia in comparison to htFibs were heterogeneous across the different patients, with minor differences (Figure S7). The observed variation in expression profiles might be related to the heterogeneous pathologies of the patients (Table S1). Contributions from individual variation were not explicitly studied here. Previous works have shown that individual variation is gene dependent, but generally much lower than the differential expression observed here for the important markers [23]. Individual variation is also expected to be mitigated by culturing of the cells. In cases when cells from several individuals were cultured in replicates under the same conditions and

length we find a tendency for the replicates for subgroups in multivariate analysis, indicating individual variation is larger than that of technical replicates, which is expected. But the main clusters separate differentially cultured cells and different cell types, evidencing that this is the dominant effect to their distinct expression profiles.

Among the studied germ cell-associated genes *STELLA*, *CD9*, *PLZF*, and *DAZL* were upregulated relative to htFibs in all cultures of spermatogonia across several different patients in comparison to htFibs (Mann-Whitney test, $P < 0.05$, compared to htFibs). The gene *POSTN* was the most homogeneously upregulated gene in this group. Furthermore among the pluripotency genes *POU5F1A*, *NANOG*, and *SOX2* were expressed to higher level and also *POSTN*, *TMEM31*, *ALDH2*, *OCT4b*, *NANOS*, *C9ORF9*, *GTSF1*, and *GDF3* (Mann-Whitney test, $P < 0.05$, compared to htFibs, Figure S6).

3. Discussion

The results presented in this study show that short-term cultured human spermatogonia (<2 weeks) can be highly enriched by CD49f MACS and matrix selection and furthermore long-term cultured. Because MACS/matrix enrichment generates a heterogeneous population of cells dominated by spermatogonia, cells with typical spermatogonial morphology were collected with a micromanipulator for microarray and real-time PCR analyses to measure gene expression profiles of adult human spermatogonia during short- and long-term culture.

We used a complex culture system with growth factors GDNF, LIF, bFGF, and EGF that supported the CD49f/matrix selected spermatogonia increasing their number by self-renewal. Using this protocol spermatogonial cells could be maintained *in vitro* for more than a year [5]. The proliferation of the cells in culture was slow. In short- and long-term cultures single as well as aligned human spermatogonia were observed. Furthermore, small grape-like aggregates of spermatogonia and also colonies of spermatogonia were present in the cultures. The aligned cells formed pairs or chains of four to eight cells, typically for more differentiated spermatogonia. In addition to the spermatogonia also some more differentiated large round cells were also present, which eventually died. This observation confirmed that MACS/matrix selection provided a heterogeneous population of cells with predominating spermatogonia and that differentiation occurred during culture. Recently, also other groups have established successful short- and long-term cultures of spermatogonia from humans with rather high proliferative capabilities [3, 6, 8, 11].

Our results show that many well-known germ cell-associated genes and pluripotency-associated genes are expressed by short-term cultured spermatogonia and also in a minor population of long-term cultured spermatogonia. The genes include *DDX4* (*VASA*) [24], *DAZL* [25–27], and *ZBTB16* (*PLZF*) [28, 29].

In addition to the established spermatogonial markers, short-term cultured spermatogonia were found to express

the ES cell-associated gene *HOOK1* in this study. *HOOK1* is a cytosolic coiled-coil protein attached to microtubules that mediates binding to cell organelles. It is found at high levels in human testes [30]. The loss of *HOOK1* gene function results in ectopic positioning of microtubular structures within the spermatids [31]. Additionally, several genes important for spermatogenesis, including *PRM1*, *PRM2*, *ODF1*, and *ODF2*, were found expressed in short-term cultured and selected spermatogonia in this study.

The majority of long-term cultured spermatogonia lost the expression of the germ cell-specific genes *VASA*, *DAZL*, and *PLZF*, but still expressed other important germ cell-associated genes including *STELLA* (*DPPA3*) and *LIN28*. Bowles et al. [32] identified the gene *DPPA3*, *developmental pluripotency-associated gene 3*, which encodes a 160-amino acid protein. In humans *DPPA3* is expressed mainly in germ cell tumors, but not in normal testes. It has been shown that mouse embryonic stem cells carrying a *STELLA* transgenic reporter can be differentiated into putative primordial germ cells (PGCs) *in vitro* [33]. It is interesting that *LIN28* and *STELLA* somehow interact. Overexpression of *LIN28* promotes the formation of *STELLA*-positive cells *in vitro* and of PGCs in chimeric embryos, and it is associated with human germ cell tumors. Perhaps the expression of these genes by the long-term cultured spermatogonia in this study reflects a more the more undifferentiated germ cell like profile.

In addition, long-term cultured spermatogonia expressed *PTN*, *SFRP1*, *POSTN*, *PTEN*, *MARCKS*, *SMAD5*, *CAR*, *ALDH2*, *CUL3*, *TSPYL1*, and *TXNIP*. *PTN*, pleiotrophin, is a heparin binding secretory growth/differentiation factor. The study by Zhang et al. [34] supported a central role of *PTN* signaling in normal spermatogenesis and suggest that interruption of *PTN* signaling may lead to sterility in mice.

The high expression of *SFRP1*, frizzled related protein 1, which was observed in the long-term cultured spermatogonia may be explained by their similarity to primordial germ cells, which undergo global demethylation [35]. *SFRP1* protein is a soluble Wnt antagonist, which has been suggested as tumor suppressor [36]. The inhibition of *WNT* transcription and activation of *NOTCH* are features of undifferentiated human embryonal carcinoma and ES cells [37], and may be mediated by the *SFRP1* gene, which is repressed in differentiating NT2/D1 cells (derived from human EC cells) after treatment with retinoic acid [38]. High expression of *SFRP1* is also observed in testis carcinoma *in situ* cells. The long-term cultured spermatogonia studied here also expressed periostin (*POSTN*), which is a member of the fasciclin family and a disulfide-linked cell adhesion protein. *POSTN* product interacts with multiple cell-surface receptors, most notably integrins, and signals mainly via the PI3-K/Akt pathway [2]. It has been shown that *POSTN* is involved in the development of various tumors [39].

The gene *CAR*, coxsackie and adenovirus receptor, functions as an adhesion molecule between Sertoli and germ cells at the Sertoli-germ cell interface [40]. Immunofluorescence staining of isolated testicular germ cells has revealed expression of *CAR* on spermatogonia, spermatocytes, round spermatids, and elongate spermatids. Similarly, Mosevitsky et al. [41] found that *MARCKS* appeared to be present at similar

level during all stages of spermatogenesis, except in mature spermatozoa.

Long-term cultured spermatogonia in this study also expressed *SMAD5*. It has been shown that expression of *SMAD5* is required for PGC development in mice [42]. *SMAD5* gene expression in the testis has been localized to stem and differentiating spermatogonia and has provided insights into the BMP regulation of spermatogenesis [43]. *SMAD5* may interact with *BMP4* and have a role in spermatogonia differentiation [44].

Also other genes found expressed by long-term cultured spermatogonia have already been confirmed during testis development and spermatogenesis. Wu et al. [45] found expression of *ALDH2*, retinoic acid-metabolizing enzymes, during mouse postnatal testis development. *CULLIN3* is a KLHL10-interacting protein preferentially expressed during late spermatogenesis [46]. *TSPYL1* is expressed by spermatogonia and mutations have been associated with disorder of male sex development and infertility [47]. *Thioredoxin-interacting protein (TXNIP)* has been associated with the formation of germ cells from embryonic stem cells in cultures with high glucose concentrations [48]. It has also been found highly expressed in adult and embryonic gonads.

Long-term cultured spermatogonia were here shown to be a heterogeneous population of cells, although still retaining the expression of several genes of pluripotency (especially *LIN28*, *SOX2*, and *NANOG*). This may reflect the *in vitro* culture condition and may be related to cell de-differentiation towards the more undifferentiated germ cell-like profile. A population of the long-term cultured cells retained and even aggravated the spermatogonial profile with the expression of germ- and pluripotency-associated genes.

Our molecular analyses of short- and long-term cultured human spermatogonia supports previous research on the culturing and propagation of human spermatogonial stem cells [9] but also raises several important issues in terms of their changes during *in vitro* culture.

While the short-term cultured spermatogonia shows the full spectrum of stem- and germ cell/differentiation-associated genes, the majority of long-term cultured spermatogonia had lost the expression of several important genes related to germ cell differentiation (e.g., *VASA*, *DAZL*, and *PLZF*) and obtained more “basal” gene expression profile. This profile may play a role in promoting the long-term proliferation of spermatogonia *in vitro*. Differential analyses of microarray expression data in this study clearly indicate that the CD49f and matrix selected short-term cultured spermatogonia were not merely fibroblasts [22] but rather showed individual germ specific expression patterns. This broad pattern was composed of germ cell type-specific and pluripotency-associated genes and may serve as transcriptional fingerprint for spermatogonia. It also became evident from the molecular analysis that a minority of cultured cells maintained a full expression spectrum of germ cell specific genes, including genes of pluripotency. This observation was possible by analysis of small samples (50 cells) with nanofluid real-time PCR technology and would have been overlooked by the inherent averaging of individual profiles when studying conventional, much larger samples. These observations

indicate that the selection criteria for spermatogonia still have to be improved. Other cell surface markers in addition to the CD49f/matrix possibly combined with manual selection have to be experimentally tested to further enrich stem cell spermatogonia during culturing. It also became evident from our study that the quality of the cultures is largely dependent on the starting material, that is, the patient's testis tissue with the pathological background. In this study only pathological tissue with limited quality was available for the experiments.

At present it is unclear if the cultured spermatogonial cells retain their full differentiation capacity of spermatogenesis after transplantation. The approach we provide to study the molecular status of *in vitro* cultured spermatogonia may be important to optimize the culture conditions and to evaluate cell plasticity in future studies. The differentiation models for spermatogenesis must be improved to unequivocally demonstrate the functional properties of the cultured human spermatogonial cells based on some new findings, such as the model of *in vitro* differentiation of long-term cultured adult human spermatogonia [9] or the organotypic slice culture system for *in vitro* spermatogenesis in mice [49]. Further studies of long-term cultured human spermatogonia are required to improve their proliferative capacities *in vitro*. This should also be accompanied by a more detailed genetic, epigenetic, and functional characterisation of *in vitro* cultured human SSCs, which have potential to become of major clinical importance.

4. Materials and Methods

4.1. Patients and Testicular Tissue. The study was performed from October 2009 until June 2011 using testicular tissue from 10 adult men with different medical backgrounds. The experiments with human material were approved by the Ethical Committee of the Medical Faculty and University of Tübingen, Chairman Professor Dr. Luft, reference number 493/2008A and Ethical Committee of the Medical Faculty of the University of Heidelberg, Chairman Professor Dr. Strowitzki, reference number S-376/2010. Informed written consent was obtained from all the human subjects. The age of the patients ranged from 20 to 84 years. Healthy, nonmalignant donated testicular tissue included heterogeneous material from patients with different medical backgrounds, including orchiectomies as part of prostate cancer treatment (1 patient), reassignment surgeries of transsexual patients after hormone therapy (6 patients), diagnostic testicular biopsy (1 patient), and a healthy testicular tissue biopsy from 1 patient with seminoma (Table S1). Histopathological examinations of the tissue used in this study were performed at the Department of Pathology (University of Tübingen) in routine diagnostics and diagnoses are presented in Table S1.

4.2. Experimental Design. In this study gene expression profiles of short-term (<2 weeks) and long-term (up to 14 months) cultured spermatogonia of ten men were analyzed. In three men (patients 184, 194, and 195) spermatogonia after one short- and three long-term cultures were analyzed based on gene expression profiles using the BioMark (Fluidigm)

for a pilot study (Table S2). In three men (patients 184, 189, and 195) spermatogonia after two short-term cultures and one long-term culture were analyzed by microarray profiling (Table S2). In seven men, including one short-term cell culture (patient 219) and six long-term cell cultures (patients 184, 191, 196, 201, 203, and 214) the microarray data were validated by real-time PCR using the BioMark (Fluidigm) system (Table S2). Spermatogonial gene expression profiles were compared to those of hESCs and htFibs.

4.3. Selection and Cultivation of hSSCs. After removing of the tunica albuginea, the obtained human testis tissues were mechanically disrupted to dissociate the tubules. The dissociated tubules were transferred for enzymatic digestion into 750 U/mL collagenase type XI (Sigma), 0.25 μ g/mL dispase II (Roche), and 5 μ g/mL DNase in HBSS buffer with Ca^{++} and Mg^{++} (PAA) for 30 minutes at 37°C, with gently mixing, to obtain a single cell suspension. Then the digestion was stopped with 10% ES cell qualified FBS, given through a 100 μ m cell strainer and centrifuged at 1000 rpm for 15 minutes. The supernatant was removed and the pellets were washed with HBSS buffer with Ca^{++} and Mg^{++} . After washing, the cells were plated into 10 cm culture dishes, coated with 0.2% gelatin (Sigma) and 2×10^5 cells per cm^2 , containing hGSC (human germ stem cell) medium with StemPro hESC medium (Invitrogen), 1% N2-supplement (Invitrogen), 6 mg/mL D+ glucose (Sigma), 5 μ g/mL bovine serum albumin (Sigma), 1% L-glutamine (PAA), 0.1% β -mercaptoethanol (Invitrogen), 1% penicillin/streptomycin (PAA), 1% MEM vitamins (PAA), 1% nonessential amino acids (PAA), 30 ng/mL estradiol (Sigma), 60 ng/mL progesterone (Sigma), 20 ng/mL epidermal growth factor (Sigma), 10 ng/mL basic fibroblast growth factor (Sigma), 8 ng/mL glial-derived neurotrophic factor (GDNF; Sigma), 100 U/mL human LIF (Millipore), 1% ES cell qualified FBS, 100 μ g/mL ascorbic acid (Sigma), 30 μ g/mL pyruvic acid (Sigma), and 1 μ L/mL DL-lactic acid (Sigma), and incubated at 37°C, 5% CO_2 for 96 hours. At day 7 the culture medium was removed and the testis cell cultures were gently rinsed with 5 mL of DMEM/F12 with L-glutamine (PAA) per plate to harvest the bound germ cells from the monolayer of adherent somatic cells by repeated pipetting with 5 mL of DMEM/F12 medium. The pooled suspension retrieved from 5 culture dishes was centrifuged at 1000 rpm for 5 minutes, suspended in 10 mL hGSC medium and plated onto one plate with collagen type I-coated 10 cm culture dish and incubated for 4 hours in the CO_2 -incubator. After the incubation the cells were rinsed again with the used medium, collected in 15 mL Falcon Tube, and centrifuged for 10 minutes at 1000 rpm. The pellet was resuspended in 10 mL of MACS buffer and centrifuged again for 5 minutes and the cells were further purified with MACS separation (Miltenyi), CD49f-FITC (α_6 -integrin; AbD serotec), and anti-FITC beads (Miltenyi). After MACS separation, positive cells from original five 10 cm dishes were plated onto 1 well of a 12-well plate on irradiated CF-1 feeder in hGSC media. Half of the media were removed every 2-3 days and replaced with half of fresh hGSC media. Under these conditions the spermatogonia proliferated quiet

heterogeneously. The cultures were split (1:2) every two to three weeks. It was important not to strongly split the cells and to keep all the time the appropriate cell number in the wells. hESCs and htFibs were collected in the same way.

4.4. Collection of Spermatogonia with a Micromanipulation System. To collect the spermatogonia for different experiments, 50 or 200 spermatogonial cells per sample/patient were collected in the first two weeks for original short-term culture or after 1, 4, 6, 8, 11, and 14 months for long-term culture experiments (see Figure S2). The cells were rinsed with the used culture media to remove the spermatogonia from the monolayer of somatic cells or feeder layer. The cells were transferred after gentle resuspension to a single cell suspension into the top of small culture dishes ($d = 3.5$ cm). The tops of the dishes were placed onto a prewarmed (37°C) working platform of an Axiovert 200 inverted microscope (Zeiss) with hydraulic micromanipulator (Narishige). At magnification 20x, the single spermatogonia were collected step by step under the criteria of typical spermatogonia morphology (only small round cells with a diameter of 6–12 μ m and high nucleus/cytoplasm ratio observed by round yellow shining nucleus and a small white cytoplasmic ring). Each spermatogonium was aspirated into the glass micropipette and transferred under visual control into the droplet of culture medium. The transfer of each spermatogonium was monitored both in the pipette and in the droplet. Fifty cells per sample probe were collected for Fluidigm analysis and 200 cells per probe for microarray analysis. After collection, the cells were transferred directly into 6.5 μ L of CellsDirect buffer (Invitrogen) for Fluidigm or 10 μ L RNA direct lyses buffer for microarray analysis.

4.5. Microarray Gene Expression Analysis. Total RNA isolated for short-term culture (during the first two weeks after matrix selection) was obtained from two independent cell cultures of spermatogonial cells from two patients (Table S1: patients 189 and 195), for long-term (up to 14 months) cultured spermatogonia from three independent cell cultures from one patient (Table S2: patient 184), from htFibs, and the hESC line H1 (WA01, WiCell) from 3 independent cell cultures and prepared using the RNeasy Mini Kit (Qiagen) followed by an amplification step with MessageAmp aRNA Kit (Ambion). Two hundred cells were collected per probe with a micromanipulator and transferred directly into 10 μ L RNA direct lysis solution and stored at -80°C . Samples were provided for analysis to the Microarray facility of University Clinic, Tübingen. Gene expression analysis was performed using the Human U133 + 2.0 Genome oligonucleotide array (Affymetrix). The raw data (CEL-files) was provided to MicroDiscovery GmbH, Berlin, Germany, for normalization and biostatistical analysis. Differential analyses were used to define lists of known germ cell-associated genes upregulated in short (SSC.short: *PROTAMIN 1*, *PROTAMIN 2*, *TNP1*, *CRISP2*, *ODF1*, *ODF2*, *ADAD1*, *PHF7*, *SPATA6*, *TMEM31*, *GSF1*, *MAEL*, *C9ORF9*, and *GTSF1*) and long-term cultured spermatogonia (SSC.long: *PTN*, *MARCKS*, *SMAD5*, *POSTN*, *ALDH1A2*, *SRFP1*, *CUL3*, *HS6ST2*,

PTEN, *LIFR*, *CCND2*, *CXADR*, and *RGS4*) when compared to testis htFibs and hESCs. Additional sets of literature-derived pluripotency-associated genes (Pluripotency: *POU5F1* (*OCT4*), *LEFTY2*, *CDX2*, *FOXO1*, *LIN28*, *HAND1*, *DPPA4*, *SOX2*, *ZIC3*, *GJA1*, and *NANOG*) and germ cell-enriched genes present in germ cells that are not specific for spermatogonia (GS genes: *TSPY1*, *DDX4* (*VASA*), *UTF1*, *CD9*, *ZBTB16* (*PLZF*), *TSPYL1*, *GPR125*, *DAZL*, *GFR α -1*, *DPPA3* (*STELLA*), *NANOS2*, *NANOS1*, *NANOS3*, *KIT*, and *LIFR*) were analyzed. In an extended approach we considered different predefined sets of genes from the literature: fibroblast-specific genes, hESC-enriched genes, and a list of genes found to be enriched in fibroblasts. All lists were extracted from the publication of Ko et al. 2010 (Figure 1k: human ES cell-enriched genes). Additionally, differential analysis of microarray data was performed by testing of germ cell-associated terms “sperm,” “testis,” “meiosis,” “germ,” and “gamete.” We investigated if these terms were annotated for the genes. To this end gene descriptions, retrieved from BioMart, were parsed for occurrences of these terms.

4.6. Microarray Data Normalization. Microarray data was imported into R Statistical Environment version 2.12.1 (2010-12-16). Data condensation was performed using Bioconductor package *affy* version 1.28.0. The condensation criteria were `bg.correct = FALSE`, `normalize = FALSE`, `pmcorrect.method = “pmonly”`, `summary.method = “medianpolish”`. Additional normalization was performed between samples using multi-*lowess* algorithm, a multidimensional extension of *lowess* normalization strategy [50]. Additional data are presented in a Supplemental Methods section.

4.7. Statistics of Microarray Data. The heatmaps and dendrograms of sample relations were used to evaluate the distances or degree of relationship between the compared experimental samples: SSCs, hESCs, and htFibs by calculating the whole assessed transcriptome, features with highest variance, or features with highest discrimination power. All heatmaps and dendrograms were created using hierarchical complete linkage clustering with Euclidean distance. The volcano plots were used to visualize fold changes and *t*-test *P* values of the comparisons of short-term and long-term cultured spermatogonia with the control groups htFibs and hESCs. The statistical significance of gene expression differences between different groups of cells (hSSCs, hESCs, and htFibs) was evaluated by different statistical tests: *F*-test, *t*-test, Pearson correlation coefficient, Wilcoxon test, and Mann-Whitney test. Significance criterion was $P < 0.05$.

4.8. Gene Expression Analyses on the Fluidigm BioMark System. To characterize the spermatogonial cells cultured under different conditions we first used the dynamic array chips (Fluidigm) to measure the expression of multiple genes in a very small number of cells. In samples from three men (Table 2 S2) spermatogonia after one short- and three long-term cultures were analyzed. In seven men (one short-term cell culture and six long-term cell cultures) microarray data were validated on the BioMark (Fluidigm) system (Table S2).

For each sample 50 spermatogonia, 50 control htFibs or 50 hESCs were manually selected from the different cell cultures with a micropipette and a micromanipulator. The pooled cells from each group were lysed, mRNA was reverse-transcribed into cDNA, which was sequence-specifically preamplified in a single tube, and the amount of targeted transcripts was quantified using TaqMan real-time PCR on the BioMark system (Fluidigm). Gene expression cell analysis of spermatogonia (50 cells per sample) in comparison with human embryonic stem cells (positive control, 50 cells per sample) and htFibs (negative control, 50 cells per sample) was performed using the BioMark Real-Time quantitative PCR (qPCR) system (Fluidigm). In all cell samples expression of up to 63 genes was measured: germ cell-specific genes (expressed in human and mouse spermatogonia): *TSPYL*, *DDX4* (*VASA*), *DAZL*, *ZBTB16* (*PLZF*), *DPPA3* (*STELLA*), *CD9*, *NANOS*, *UTF1*, *GFR α -1*, *KIT*, *KITLIG*, *DNMT3B*, *GPR125*, and *LIFR*; pluripotency-associated genes: *POU5F1* (*OCT4A*), *POU5F1* (*OCT4B*), *LIN28*, *NANOG*, *SOX2*, *GDF3*, *LIN28B*, *CDH1*, *TDGF1*, *TERT*, *MYC*, *STAT3*, *KLF4*, and *REX1*; germ cell-enriched genes (known as germ cell genes but normally are not used to characterize spermatogonia): *PROTAMIN1* (*PRM1*), *PROTAMIN 2* (*PRM2*), *TNPI*, *CRISP2*, *ODF1*, *ODF2*, *ADAD1*, *PHF7*, *SPATA6*, *TMEM31*, *GSF1*, *MAEL*, *C9ORF9*, *GTSF1*, *PTN*, *MARCKS*, *SMAD5*, *POSTN*, *ALDH1A2*, *SRFP1*, *CUL3*, *HS6ST2*, *COLEC12*, *C9ORF9*, *TSPYL1*, *RGS4*, *PTEN*, and *SOX9*; fibroblast-associated genes: *COL1A1*, *COL1A2*, *FIBRONECTIN1*, and *VIMENTIN*; and the housekeeping genes *CTNNB1*, *HNBS*, and *GAPDH*.

For preamplification the inventoried TaqMan assays (63x, Applied Biosystem) were pooled to a final concentration of 0.2x for each of the 63 assays. Cells to be analysed were harvested directly into 9 μ L RT-PreAmp Master Mix (5.0 μ L CellsDirect 2x Reaction Mix (Invitrogen); 2.5 μ L 0.2x assay pool; 0.2 μ L RT/Taq Superscript III [Invitrogen]; 1.3 μ L TE buffer). The harvested cells were immediately frozen and stored at -80°C . Cell lysis and sequence-specific reverse transcription were performed at 50°C for 15 min. The reverse transcriptase was inactivated by heating to 95°C for 2 min. Subsequently, in the same tube, cDNA went through limited sequence-specific amplification by denaturing at 95°C for 15 s and annealing and amplification at 60°C for 4 min for 14 cycles. These preamplified products were diluted 5-fold prior to analysis with Universal PCR Master Mix and inventoried TaqMan gene expression assays (ABI) in 96.96 dynamic arrays on a BioMark system. Each sample was analyzed in three to four technical replicates. C_t values obtained from the BioMark system were transferred to the GenEx software (MultiD) for analysis. Additional information on GenEx analysis is presented in a Supplemental Methods section.

4.9. Immunocytochemistry. To characterize human spermatogonia, we examined the expression of a panel of cell-specific proteins including CD49f, Dazl, VASA, STELLA, and the somatic marker VIMENTIN.

Antibodies and Staining. The following primary antibodies were used: mouse monoclonal biotinylated anti-CD49f (BioLegend), mouse monoclonal anti-Dazl (AbD Serotec),

goat polyclonal anti-VASA (R&D Systems), rat monoclonal anti-STELLA (R&D Systems), and mouse monoclonal anti-VIMENTIN (Dako). The following secondary antibodies were used: Cy3 conjugated Streptavidin (Dako), Alexa Fluor-488 conjugated goat anti-mouse IgG (Molecular Probes), Cy3 conjugated rabbit anti-goat IgG (Dianova), and Cy3 conjugated goat anti-rat IgG (Dianova). All staining were costained with DAPI.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Thomas Skutella Sabine Conrad conceived and designed the experiments. Sabine Conrad, Hossein Azizi, Maryam Hatami, Michael Bonin, and Thomas Skutella performed the experiments. Mikael Kubista, Michael Bonin, Sabine Conrad, and Thomas Skutella analyzed the data. Michael Bonin, Jörg Hennenlotter and Markus Renninger contributed reagents/materials/analysis tools. Sabine Conrad and Thomas Skutella wrote the paper.

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Review Article

High-Throughput Analysis of Ovarian Granulosa Cell Transcriptome

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The quality of follicular oocytes depends on interactions with surrounding granulosa cells. Development of molecular techniques and methods enables better understanding of processes underlying mammalian reproduction on cellular level. The success in reproductive biology and medicine in different species depends on reliable assessment of oocyte and embryo viability which presently mainly bases on embryo morphology. Although successful pregnancies have been achieved using this approach, its precision still should be improved and completed with other, more objective, and accurate assessment strategies. Global profiling of gene expression in follicular cumulus cells using microarrays is continuously leading to the establishment of new biomarkers which can be used to select oocytes with highest developmental potential. Even more potential applications and greater precision could be achieved using next generation sequencing (NGS) of granulosa and cumulus cell RNA (RNA-seq). However, due to the high cost, this method is not used as frequently as microarrays at the moment. In any case, high-throughput technologies offer the possibilities and advantages in ovarian somatic cell analysis on scale that has not been noted so far. The aim of this work is to present current directions and examples of global molecular profiling of granulosa cells and underline its impact on reproductive biology and medicine.

1. Introduction

Follicles in mammalian ovaries undergo regular changes including recruitment, expansion, maturation, rupture, development into corpus luteum, and final atresia. All those processes are controlled by gonadotropins secreted from the pituitary which administrate signaling networks between the oocyte and the somatic cells in the ovary. Two somatic cell types can be distinguished in the follicle: the mural granulosa cells lying on the basal membrane of the follicular wall and cumulus granulosa cells (CCs) surrounding oocyte. These cells have common origin at early follicular stage but differentiate into two subpopulations in the course of follicular development, up to the preovulatory stage. Cumulus cells stay close to the oocyte forming gap junction projections which allow transport of metabolites between the somatic cells and the oocyte. The oocyte secretes different signaling factors that control and influence proliferation and the differentiation of cumulus cells (Figure 1). Oocyte and cumulus cells form the cumulus-oocyte complex (COC) that

remains intact during ovulation up to fertilization. Mural granulosa cell layer remains in less tight contact with oocyte due to the distance and is responsible for the steroidogenic activity of the maturing follicle. Mural granulosa cells express receptors for LH necessary for triggering the final maturation of the follicle and ovulation [1, 2].

The advent and development of “omics” field, such as genomics, transcriptomics, proteomics, and metabolomics, is continuously changing our understanding of mammalian physiology and approaches towards solving particular health problems [3]. In addition to studies aiming at better understanding of basis and processes of mammalian folliculogenesis, increasing interest and significance of high-throughput techniques such as microarrays, next generation sequencing, or single cell genomics is also observed in reproductive medicine to evaluate quality of oocytes and embryos [4].

Transcriptome is the RNA content of the cell [3] while transcriptomics relates to study of transcriptomes and their functions. It serves as a tool to analyse a large number of genes in parallel and bases on assumption that genes involved in

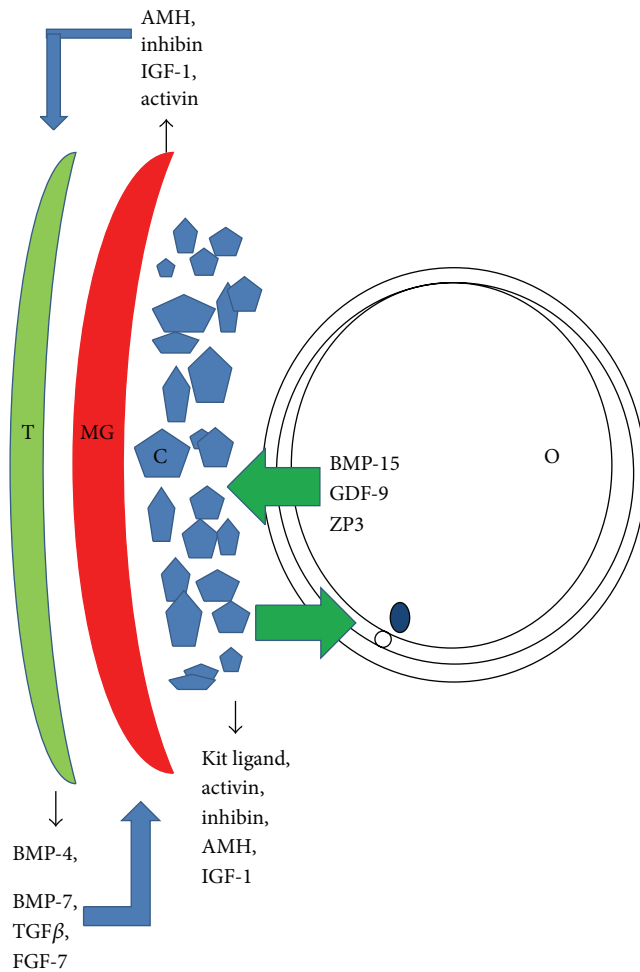


FIGURE 1: Schematic illustration of mutual interactions (via paracrine growth factors) between somatic cells and oocyte in ovarian follicles (T: theca cell layer, MG: mural granulosa cell layer, C: cumulus cells, and O: oocyte).

specific processes are expressed together and genes showing similar expression patterns may be functionally linked and regulated by similar genetic control mechanisms. Patterns of gene expression levels deliver information about functional status of the cell [5].

The basic and most widely used approach for transcriptome analysis is microarray hybridization. Currently, several microarray systems are used among which Affymetrix and Illumina platforms are the most popular ones. Illustration of main steps of granulosa cell RNA preparation for microarray analysis is presented in Figure 2.

Next generation sequencing (NGS) is an alternative approach to study qualitative and quantitative RNA content of the cells (RNA-seq). NGS delivers huge amount of data which, after bioinformatic analysis, can be translated into biological knowledge. Although the cost of NGS analysis has been significantly reduced in recent years, it is still considerable which limits application of this technology in routine clinical practice. Nevertheless, this method is being increasingly used in research. It is assumed that NGS, not microarrays, will be the basic tool for genomic and transcriptomic

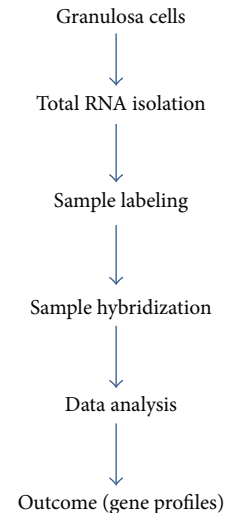


FIGURE 2: Schematic illustration of preparation of RNA isolated from granulosa cells for transcriptome analysis.

analysis in the future as its potential to deliver biologically relevant information is much greater than in case of approach based on chips.

In this work, current applications, directions, and examples of using high-throughput technologies for investigation of ovarian granulosa cell transcriptome are presented.

2. Transcriptomic Analysis of Granulosa Cells to Determine Factors Involved in Follicular Development

Large number of studies have been carried out to identify the mechanisms controlling folliculogenesis. Early folliculogenesis (from follicle formation of the resting pool to the preantral stage) is particularly important as it affects and regulates the size of the resting primordial follicle pool and the fate of the follicles, which in turn influences reproductive life span and fertility [1, 2]. Ovarian follicle is a mixture of different cell types. Cell-specific gene expression driving early folliculogenesis is still not completely known. To elucidate the drive factors of follicular development Bonnet et al. [6] successfully developed laser capture microdissection (LCM) technique coupled with microarray to isolate and investigate very small sized follicles from sheep ovary. Such approach ensured pure cell populations (granulosa cells and oocytes), the transcriptome of which was profiled using bovine chip. The study revealed 1050 transcripts specific to the granulosa cells and 759 specific to the oocyte (comparison of oocyte versus follicular cell transcriptomes). This work, for the first time, demonstrated the global gene expression pattern in specific follicular compartments using high-throughput technology. Obtained data were basis for functional analysis related to processes critical for early folliculogenesis.

To determine crucial factors regulating the gonadotropin-independent and -dependent follicle growth stage and to facilitate development of a culture system for early growing

follicles, DNA microarray analysis of mouse ovaries recovered at 7, 10, 13, 16, and 19 days of age was performed [7]. This study showed strong intensity of zona pellucida glycoproteins, bone morphogenic protein-15 (BMP-15), and growth differentiation factor (GDF-9) in 7-day-old mice, which gradually declined in 19-day-old mice. KIT, KIT ligand, anti-Müllerian hormone (AMH), and platelet-derived growth factor (PDGF), which are secreted by granulosa cell secreted factors, also showed relatively high expression. This work contributed to the understanding of factors involved in follicular development and this knowledge may be used for establishment of new in vitro systems for the culture of follicles with potential medical application in severely affected patients.

Using The NimbleGen platform with high-density expression arrays, Batista et al. [8] compared the transcriptomes of granulosa-like cells overexpressing, or not, FOXL2, one of the earliest ovarian markers which, together with its targets, can serve as a model to study ovarian development and function in normal and pathological conditions. It was demonstrated that mediators of inflammation, apoptotic and transcriptional regulators, genes involved in cholesterol metabolism, and genes encoding enzymes, and transcription factors involved in reactive oxygen species detoxification were upregulated. On the other hand, the transcription of genes involved in proteolysis, signal transduction as well as in transcription regulation was shown to be down-regulated by FOXL2. Potential target promoters activated and repressed by FOXL2 were discriminated by bioinformatic analysis.

Using genome-wide analysis of DNA microarray data sets based on samples from periovulatory ovaries, Kawamura et al. [9] found increases in natriuretic peptide precursor type C (NPPC-gene encoding C-type natriuretic peptide, CNP) transcripts in granulosa cells during preovulatory follicle growth in mice and a rapid decline induced by the preovulatory LH/hCG stimulation. Ovarian CNP content was decreased upon treatment of preovulatory animals with hCG. NPPC mRNA was predominantly expressed in mural granulosa cells exhibiting similar regulation following gonadotropin treatment, in isolated ovarian cells.

Bonnet et al. [10] identified the genes differentially expressed in pig granulosa cells in the course of the terminal ovarian follicle growth, to obtain a comprehensive view of these mechanisms. In the first step, specific microarray was developed using cDNAs from suppression subtractive hybridization libraries (345 contigs) obtained by comparison of three follicle size classes: small, medium, and large antral healthy follicles. In the next step, a transcriptomic analysis using cDNA probes from these three follicle classes identified differentially expressed transcripts along the terminal follicular growth and genes predictive of size classes (Table 1). The data analysis allowed identifying gene networks important for terminal follicular development.

In order to elucidate the differentiation status and responsiveness to gonadotropin stimulation in ER β -null mice, recently, Binder et al. [11] isolated preovulatory granulosa cells from wild-type and ER β -null mice using laser capture microdissection. The aim was to examine the genomic transcriptional response downstream of pregnant mare serum gonadotropin (mimicking FSH) and pregnant mare serum

gonadotropin/human chorionic gonadotropin (mimicking LH) stimulation. This approach allowed a comparison of in vivo granulosa cells at the same stage of development from both wild-type and ER β -null ovaries. ER β -null granulosa cells showed altered expression of genes known to be regulated by FSH (Akap12 and Runx2) as well as not previously reported (Arnt2 and Pou5f1) in wild-type granulosa cells. The analysis also identified more than 300 genes not previously associated with ER β in granulosa cells (Table 1).

Small RNAs including microRNAs (miRNAs) are now considered as important regulators of follicular development [20]. Using small RNA sequencing (small RNA-seq) Velthut-Meikas et al. [21] determined the miRNA profile of the two intrafollicular somatic cell types: mural and cumulus granulosa cells isolated from women undergoing controlled ovarian stimulation and in vitro fertilization. In total, 936 annotated and 9 novel miRNAs were identified. Ninety of the annotated miRNAs were differentially expressed between mural granulosa cells and cumulus cells. Bioinformatic analysis revealed that TGF β , ErbB signaling, and heparan sulfate biosynthesis were targeted by miRNA in both granulosa cell populations, while extracellular matrix remodeling, Wnt, and neurotrophin signaling pathways were targeted by a miRNA in mural granulosa cells.

3. Transcriptomic Analysis of Granulosa Cells to Evaluate Oocyte and Embryo Quality

The fact that cumulus cells are in close communication with oocyte via gap junctions and local paracrine factors suggests that their analysis may give reliable information about the oocyte itself. Cumulus cells can be easily collected without compromising the oocyte, which makes them attractive targets for studies on noninvasive biomarkers of oocyte developmental competence [4].

Gene expression profiling of mural rat granulosa cells with Affymetrix rat whole genome array revealed that the most differentially expressed gene, lysyl oxidase, may be a candidate biomarker of oocyte health and can be used for the selection of good quality oocytes for reproductive biology procedures [13] (Table 1).

Using microarrays Assidi et al. [15] aimed at identifying markers of oocyte competence that are expressed in bovine cumulus cells. Candidate genes expressed in cumulus cells which could be valuable and indirect markers of oocyte competence were hyaluronan synthase 2 (HAS2), inhibin betaA (INHBA), epidermal growth factor receptor (EGFR), gremlin 1 (GREM1), betacellulin (BTC), CD44, tumor necrosis factor-induced protein 6 (TNFAIP6), and prostaglandin-endoperoxide synthase 2 (PTGS2). These biomarkers were proposed to be potential candidates to predict oocyte competence and to select higher-quality embryos for transfer (Table 1). Using bovine model Bettegowda et al. [14] identified differences in RNA transcript abundance in cumulus cells harvested from oocytes of adult versus prepubertal animals (characterized by poor oocyte quality) by microarray analysis. It was revealed that four genes encoding for the lysosomal cysteine proteinases cathepsins B, S, and Z displayed greater transcript abundance in cumulus cells surrounding

TABLE 1: Granulosa cell gene expression profiling in non-human species (↑ and ↓ denote up- and downregulated genes, resp.).

Species	Type of cell	Quantity of material analysed	Kind of assay	Gene profiles/outcome	Reference
Mouse	Preovulatory granulosa cells from wild-type and ERβ-null mice	Captured cells from 2-3 animals of the same genotype were pooled, with at least 3 pools collected per cell type	Affymetrix Mouse Genome 430 2.0 GeneChip arrays	<i>Tigf3</i> , <i>Ak7</i> , <i>Arnt2</i> , <i>Nid2</i> , <i>Eya4</i> , <i>Mbl2</i> , <i>Ooep</i> , <i>Ptgs</i> , <i>Oogl</i> , <i>Pou5f1</i> , <i>Trim61</i> , and <i>Tmem182</i> ↑ in PMSG primed ERβ-null mice granulosa cells versus PMSG primed wild-type mice granulosa cells. <i>Epha5</i> , <i>Ank1</i> , <i>Me2</i> , <i>Reln</i> , <i>Cbs</i> , <i>Cdh2</i> , <i>Ces1d</i> , <i>Mpp7</i> , <i>an Susd4</i> ↓ in PMSG primed ERβ-null mice granulosa cells versus PMSG primed wild-type mice granulosa cells	Binder et al., 2013 [11]
	CCs from noncompetent antral oocytes CCs from competent antral oocytes	Total 638 COCs Total 1769 COCs	Illumina Sentrix arrays	<i>Has2</i> , <i>Ptx3</i> , <i>Tnfrsf6</i> , and <i>Ptgs2</i> ↓ <i>Amlh</i> ↑ in noncompetent versus competent oocytes	Vigone et al., 2013 [12]
Rat	Mural granulosa cells from normal developmental competence oocyte-collected follicles (NDC)	4 samples	Affymetrix GeneChip arrays Rat 230.2.	<i>Lox</i> , <i>Ngfrap1</i> ↑ <i>Ggbit2</i> ↓ in NDC versus PDC	Jiang et al., 2010 [13]
	Mural granulosa cells from poor developmental competence oocyte-collected follicles (PDC)	4 samples			
Pig	Small, medium, and large antral follicles	Pooled granulosa cells from follicles of three different size classes. Four samples from small follicles, five samples from medium and large follicles	Microarray	<i>Hadhb</i> , <i>Psmc2</i> , <i>Gstl1</i> , <i>Ctst</i> , <i>Hspa8</i> , <i>Mgstl</i> , <i>Erp29</i> , <i>Gart</i> , <i>Cyb5</i> , and <i>Cct1</i> ↑ in large follicles versus small and medium follicles <i>Gstl1</i> , <i>Pkm2</i> , <i>Tubal1b</i> , <i>Calu</i> , <i>Cfl1</i> , <i>Dag1</i> , <i>Tubb5</i> , <i>Eef1a</i> , and <i>Rpsl7</i> ↑ in small follicles versus medium and large follicles <i>Ctstb</i> , <i>Ctss</i> , and <i>Ctssz</i> ↑	Bonnet et al., 2008 [10]
Bovine	Cumulus cells from adult and prepubertal oocytes	Pools of cumulus cells (<i>n</i> = 4) collected from adult and prepubertal animals	Bovine cDNA array (Gene Expression Omnibus platform GPL325)	in cumulus cells collected from oocytes with low developmental competence versus those collected from oocytes with high developmental competence	Bettegowda et al., 2008 [14]
Bovine	COCs from 3 to 8 mm follicles	4 samples	Homemade microarray performed using the VersArray ChipWriter Pro	<i>Has2</i> , <i>InhibA</i> , <i>Egfr</i> , <i>Grem1</i> , <i>Birc</i> , <i>CD44</i> , <i>Tnfrsf6</i> , and <i>Ptgs2</i> , markers of oocyte competence, expressed in CCs	Assidi et al., 2008 [15]

TABLE 2: Gene expression profiling of human granulosa and cumulus cells.

Type of cell	Assay	Gene profiles/outcome	Reference
Granulosa cells from aspirated follicular fluid	Microarray	<i>FDX1</i> , <i>CYP19A1</i> , <i>CDC42</i> , <i>SERPINE2</i> , and <i>3bHSD1</i> , positively correlated with oocyte developmental competence	Hamel et al., 2008 [16]
Cumulus cells	Whole Human Genome Oligo Microarray 4x44K (Agilent Technologies)	<i>RGS2</i> , related to oocyte developmental competence	Feuerstein et al., 2012 [17]
Cumulus cells	Affymetrix HG-U133 Plus 2.0 array	<i>BCL2L1</i> , <i>PCK1</i> , and <i>NFIB</i> , significantly correlated with embryo potential	Assou et al., 2008 [18]
Cumulus cells from gonadotropin stimulated patients	Human Genome U133 Plus 2.0 microarrays	<i>NPY1R</i> , <i>GM2A</i> , <i>GREM1</i> <i>OSBPL6</i> , <i>STC2</i> , and <i>PTX3</i> , correlated with embryo quality	Assou et al., 2013 [19]

oocytes harvested from prepubertal animals. Functional analysis indicated the role for cumulus cell cathepsins in compromised oocyte competence and resulted in conclusion that cumulus cell cathepsin transcript abundance may be predictive of oocyte quality (Table 1).

These close relations between oocyte and cumulus cells are of particular significance in assisted reproduction procedure. Single embryo transfer is considered the most appropriate way to reduce the frequency of multiple pregnancies following in vitro fertilisation. However, selecting the embryo for single transfer embryo with the highest chances of pregnancy remains a difficult challenge since morphological and kinetics criteria provide poor prediction of both developmental and implantation ability. The oocyte-cumulus interaction through the expression of specific genes helps the oocyte to acquire its developmental competence. Using microarrays Feuerstein et al. [17] attempted to determine genes related to oocyte developmental competence. Gene expression of oocytecumulus was studied according to the nuclear maturity of the oocyte (immature versus mature oocyte) and to the developmental competence of the oocyte (ability to reach the blastocyst stage after fertilisation). Microarray analysis data delivered 308 differentially expressed genes out of which 8 genes were selected according to oocyte developmental competence for further validation. Three of these 8 selected genes were validated as potential biomarkers (*PLIN2*, *RGS2*, and *ANG*). Finally, *RGS2*, known as a regulator of G protein signalling, was the only gene among selected candidates biomarkers of oocyte competence which covered many factors of variability (Table 2).

Using human Genome U133 Plus 2.0 microarrays Ouandaogo et al. [22] performed an analysis of the genes expressed in human cumulus cells obtained from patients undergoing intracytoplasmic sperm injection. Cumulus cells samples were isolated from oocyte at germinal vesicle, stage metaphase I, and stage metaphase II. Differentially over-expressed genes between the three cumulus cells categories were identified. The mentioned study demonstrated a specific signature of gene expression in cumulus cells obtained from MII oocyte compared with germinal vesicle and metaphase I. As concluded by the authors gene expression profile, which

is specific of MII mature oocyte, may be used as predictor of oocyte quality. Although the study failed to list specific markers of oocyte quality, it anyway underlined the distinct gene signature of individual CC samples isolated from oocytes at different stages of development. To assess such signatures is a first necessary step to qualify the cumulus cell status as competent or incompetent.

As the maturation conditions of human cumulus-oocyte complexes might affect gene expression in both oocyte and cumulus cells Ouandaogo et al. [23] compared the transcriptome profiles of cumulus cells isolated from in vivo and in vitro matured COC at different nuclear maturation stages. In this study microarray technology was used to analyse the global gene expression to compare the expression profiles of CCs from COC at different nuclear maturation stages following IVM or in vivo maturation. Afterwards, selected genes were validated by qPCR. It was found out that, in CCs isolated after IVM, genes related to cumulus expansion and oocyte maturation, such as *EREG*, *AREG*, and *PTX3*, were down-regulated, while cell cycle-related genes were upregulated in comparison with CCs from in vivo matured COC from polycystic ovary syndrome and normal responder patients. Using Affymetrix Gene Chip Mouse Genome 430 2.0 array Kind et al. [24] observed that 1593 genes were differentially expressed, with 811 genes upregulated and 782 genes downregulated in mouse IVM compared with IVV cumulus cells; selected genes were validated by real-time reverse transcription-polymerase chain reaction.

Using DNA microarrays Haouzi et al. [25] studied the LH/hCGR gene expression in cumulus cells surrounding oocytes in patients undergoing controlled ovarian hyperstimulation (COS) before ICSI and related it to other ovarian hyperstimulation quality parameters. The transcriptome analysis of CC indicated a variable expression of LH/hCGR among the patients and intrapartients. LH/hCGR mRNA expression was negatively correlated with serum estradiol level on the day of hCG administration. Eighty-five genes, playing role mainly in steroid metabolism and in the ovulation process (including *TNFAIP6*), were significantly modulated between cumulus cells from patients with a high and a low LH/hCGR expression. No significant differences in

LH/hCGR gene expression profile between COS protocols were observed.

Recently, Assou et al. [19] characterized and compared gene expression profiles in cumulus cells of periovulatory follicles obtained from patients stimulated with HP-hMG or rFSH in a GnRH antagonist cycle and studied their relationship with in vitro embryo development, using Human Genome U133 Plus 2.0 microarrays. Genes upregulated in HP-hMG-treated CCs are involved in lipid metabolism (GM2A) and cell-to-cell interactions (GJA5). On the other hand, genes upregulated in rFSH-treated CCs play role in cell assembly and organization (COL1A1 and COL3A1). It was demonstrated that some genes specific to each gonadotropin treatment (NPY1R and GM2A for HP-hMG; GREM1 and OSBPL6 for rFSH) were associated with day 3 embryo quality and blastocyst grade at day 5, whereas others (STC2 and PTX3) were linked to in vitro embryo quality in both gonadotropin treatments. Embryo and blastocyst quality were assessed daily by the embryologists until 5 days after oocyte retrieval. Top quality 8-cell embryos at day 3 were subjected to blastocyst outcome analysis at day 5 (Table 2).

In study of Hamel et al. [16] hybridization data analysis discriminated 115 genes associated with competent human follicles (leading to a pregnancy). Selected candidates were confirmed by Q-PCR: 3-beta-hydroxysteroid dehydrogenase 1, ferredoxin 1, serine (or cysteine) proteinase inhibitor clade E member 2, cytochrome P450 aromatase, and cell division cycle 42 (Table 2).

Global gene expression analysis performed by Vigone et al. [12] showed that developmentally incompetent and competent CCs share similar transcriptomes, with the exception of 422 genes, 97.6% of which were downregulated in incompetent versus competent CCs. This demonstrated that developmental incompetence or competence of antral oocytes can be predicted using transcript markers expressed by their surrounding CCs (i.e., Has2, Ptx3, Tnfrsf6, Ptg2, and Amh) (Table 1). Using a combined microarray and quantitative reverse-transcription polymerase chain reaction approach Lager et al. [26] found a set of 12 genes predictive of pregnancy outcome based on their expression levels in CCs.

Using microarrays, recently, Papler et al. [27] analyzed surrounding mature oocytes that developed to morulae or blastocysts on day 5 after oocyte retrieval. The analysis revealed 66 differentially expressed genes between cumulus cells of modified natural IVF and controlled ovarian hyperstimulation cycles. Further gene analysis showed that the oxidation-reduction process, glutathione metabolic process, xenobiotic metabolic process, and gene expression were significantly enriched biological processes in modified natural in vitro fertilization cycles. The study of Assou et al. [18] showed that the expression of BCL2L1, PCK1, and NFIB in CCs is significantly correlated with embryo potential and successful pregnancy (Table 2).

4. Summary

Infertility is a civilization disease which affects a significant number of couples of reproductive age. It is also a serious medical and financial challenge which has been approached

for years with increasing efficiency and with application of different methods and strategies. The progress in the field of reproductive biology strongly depends on basic research and better understanding of processes regulating follicular development and oocyte maturation. This knowledge can be then translated into clinical practice which enables solving of particular problems of fertility. As presented in this review, transcriptomics is increasingly applied to the gamete and embryo assessment which leads to establishment of biomarkers linked to oocyte and embryo quality. "The omics" offer much more reliable and objective way to assess viability of oocytes and embryos in comparison to conventional morphological evaluation. However, some problems related to the wide application of these methods in clinical practice are still faced. Out of them, duration of analysis and relatively high costs should be mentioned as main obstacles. Therefore, a combination of conventional morphological criteria of evaluation with new, fast, inexpensive, easy-to-use, and noninvasive high-throughput techniques is expected to ensure the progress and efficiency of infertility diagnostics and treatment [3]. Development of high-throughput methods will also be beneficial for solving fertility problems in patients suffering from premature ovarian failure and ovarian impairment due to adjuvant therapy for cancer. It also should be mentioned that not only further achievements in the field of transcriptomics but also proteomics (analysis of cell proteome) will be critical for progress in reproductive biology and medicine.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Cumulative Delivery Rate after Providing Full Reimbursement In Vitro Fertilization Programme: A 6-Years Survey

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Since 1983, Slovenia has been offering well-established, successful, and fully reimbursed IVF programme to infertile couples. On the grounds of data gathered at the Slovenian IVF units we aimed to determine whether the fully accessible IVF treatment system can provide notable success considering cumulative delivery rate (cDR). Longitudinal analysis of getting cDR was performed in 810 IVF cycles of 395 couples who for the first time attended the IVF programme in year 2006 and were followed until year 2012. We calculated the actual and the optimistic cDR. In women aged <38 years the actual cDR was 54% and optimistic DR was 83%, respectively. In women aged ≥38 years the actual cDR was 24 % and optimistic cDR was 27%. These results enable us to report that prospects of the treatment for the women aged <38 years, if they undergo all 6 available IVF cycles, are very positive and quite comparable to the chances of spontaneous conception. Even in older patients it is beneficial to repeat the IVF procedures. Therefore we consider the existing infertility treatment system in Slovenia as an example of good medical practice with high level of beneficence offered to the patients.

1. Introduction

Since 1983, Slovenia has been offering well-established and successful IVF treatment programmes to infertile couples. According to Europe IVF monitoring (EIM), Slovenia achieves high rankings in terms of IVF treatment accessibility (number of IVF cycles, ICSI cycles, and frozen-thawed cycles per million inhabitants), while in 2009, the percentage of IVF-related deliveries (4,5%) was even amongst the highest percentages in Europe [1].

In Europe the IVF treatment is performed 2.5 times more frequently compared to the United States [1, 2]. This difference is probably due to different funding systems [3].

In Slovenia, the IVF treatment programmes offer full access to infertile couples. Under the term “full access,” Slovenian medical assurance system recognises (under compulsory and supplementary health insurance) the broadest concept of this term, offering the treatment notwithstanding

the treatment prognosis, including full reimbursement of costs of IVF cycles by the state, and allowing reinclusion into the programme after delivery. The limitations of treatment are set only in terms of female age (up to 43 years) and the number of cycles (6 cycles for the first delivery and additional 4 cycles on reinclusion into the programme after the first delivery [4]. These limitations reasonably draw a line between effective and ineffective (futile) treatment, since it has been demonstrated that delivery rate (DR) decreases to the level of futile assistance after the female age of 43 years [5, 6].

Based on our own experience, Slovenia introduced new legislation regarding infertility treatment and biomedically assisted procreation in year 2000 [7]. Before adoption in the Parliament, the legislation had been discussed and drafted by a group of infertility and legal experts and has not been supplemented or amended nor challenged by any person before the Constitutional Court of the Republic of Slovenia since its adoption in year 2000. Together with continued

and successful practice of fully reimbursed IVF treatment, this shows efficiency as well as stability of the regulation in question.

In other European countries there are different approaches to IVF treatment in terms of financial access: the policies regarding the level of coverage and the number of cycles covered by the state differ significantly. Some of the countries with full coverage of IVF treatment include Belgium (6 cycles), France (4 cycles), The Netherlands (3 cycles), and Sweden (various number of cycles). "Full coverage" in this context is defined as 100% coverage of at least one cycle of IVF on a national basis. According to Berg Brigham et al. [8], France is supposed to be the only European country where a treatment cycle constitutes a fresh stimulated cycle, while any subsequent transfer using frozen embryos is not considered as a new cycle. We may add, however, that the same policy is also applied in Slovenia.

Since the European Parliament called on member states to ensure the right of couples to universal access to infertility treatment [9] and since the European Society of Human Reproduction and Embryology (ESHRE) concluded that reimbursement policies can have a significant impact on the accessibility and use of ART treatments [3, 10–14], we consider the existing infertility treatment system in Slovenia with the above mentioned characteristics of "full access" as an example of good medical practice which enables the infertile couples to repeat the IVF cycles and in this way to improve the cumulative delivery rate (cDR).

The most often performed analysis to determine the success rates (of IVF clinics or rates on the national level) includes cross-sectional statistics. Also the national reports to the European IVF Monitoring (EIM) at ESHRE are prepared in terms of pregnancy outcome per started cycle or embryo transfer [1]. However, such statistics have only a limited value for individual patients. The DR per each cycle is not very indicative in terms of overall success prognosis of each couple. Couples are usually more interested in their realistic overall prospects on the treatment and their actual chances to conceive a child, if they undergo all IVF cycles. At the same time it is also important for the state to evaluate actual outcomes of its accessible IVF treatment system with full reimbursement, acknowledging all accessible cycles. It may be concluded therefore that better evaluation of IVF treatment should be done by performing longitudinal analysis including the data on repeating IVF cycles.

Therefore the aim of our longitudinal study was to determine cDRs by repeating IVF cycles in infertile couples included into our programmes, also showing cDRs per specific female age groups and different ovarian responses to hormonal stimulation. Additionally we also aimed to critically discuss gathered cDR data in relation to the basic principles of Slovenian IVF treatment system, trying to determine the level of beneficence of current regulation.

2. Materials and Methods

2.1. Infertile Couples and IVF Cycles. In this longitudinal study we analysed 810 IVF cycles in 395 couples who

for the first time attended the IVF program of IVF Unit of Department of Obstetrics and Gynaecology, University Medical Centre Ljubljana, in year 2006 and were followed until year 2012. The repeated IVF cycles were performed at infertility centres in Ljubljana, Maribor, and Postojna. We followed all patients until the end of year 2012 since we expected that in this time period all patients would have completed all 6 available IVF cycles. We aimed to find out whether the fully accessible IVF treatment system in our country can deliver notable success considering cDR for both the individual couple and the state considering the female age and ovarian response to the hormonal stimulation. The patients were divided according to their age (women aged <38 years and women aged ≥38 years) and according to the response to ovarian stimulation (poor: ≤3 retrieved oocytes and normal: >3 retrieved oocytes). Poor prognosis is known to be related to the increasing female age [5, 6] and poor ovarian response to ovarian stimulation by gonadotropins [15–17].

2.2. Indications of Infertility. In the observed study population, the main indications for IVF treatment were tubal factor infertility, endocrine factors of infertility, unexplained infertility, endometriosis, male factor infertility, or combinations of these.

2.3. Ovarian Hormonal Stimulation. For ovarian stimulation the combination of gonadotropins and GnRH agonist was used preferentially. Controlled ovarian stimulation (COH) was performed with either recombinant follitropin alfa or beta (rFSH) or urinary HMG. The starting dose was administered individually. For oocyte maturation HCG was administered and oocyte retrieval was performed 36 h after HCG administration. At most two embryos were transferred. All supernumerous embryos were cryopreserved. For luteal supplementation progesterone intravaginally was used.

2.4. Longitudinal Analysis, Cumulative Pregnancy, and Delivery Rates. We calculated the cumulative pregnancy rate (cPR) and cumulative delivery rate (cDR) for all couples undergoing the first IVF treatment in year 2006 and all their subsequent repeated fresh or frozen-thawed cycles until the end of year 2012. Such follow-up period was considered to be sufficient for all patients to undergo 6 available cycles during this time period. To estimate cPR and cDR we included the repeated IVF cycles of these couples at our IVF unit or other Slovenian IVF units, at the University Medical Centre in Maribor or at the IVF Unit of Gynaecological Hospital in Postojna.

In this longitudinal analysis we wanted to identify the benefit of repeating IVF cycles in terms of achieving better cDR. We also wanted to evaluate the importance of repeating cycles in patients with poor prognosis [5, 6, 15–17].

The women were divided according to age (<38 and ≥38 years) and according to the response to ovarian stimulation (≤3 retrieved oocytes and >3 retrieved oocytes).

The cPRs and cDRs were determined by two approaches, as actual cPR/cDR and as optimistic cPR/cDR. Actual

TABLE 1: Delivery rates (DRs) by women's age according to consecutive number of treatment IVF cycle.

	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
DR (<i>n</i>)	0.28 (109)	0.22 (50)	0.20 (20)	0.14 (7)	0.09 (2)	0.27 (4)
DR < 38 (<i>n</i>)	0.30 (97)	0.25 (45)	0.25 (20)	0.19 (7)	0.13 (2)	0.40 (4)
DR ≥ 38 (<i>n</i>)	0.17 (12)	0.12 (5)	0 (0)	0 (0)	0 (0)	0 (0)

cPRs/cDRs were calculated as cumulative numbers of pregnancies and deliveries in observed group of women without any methodological modification in relation to dropout patients. We further calculated the optimistic cDR, since in the actual study population we acknowledged that certain dropout of the patients occurred irrespective of the fully reimbursed system being available in Slovenia. In optimistic cPR/cDR a statistical correction according to the dropout was performed. A correction assumes that women who did not return for subsequent IVF cycles would have the same chance of delivery as those who remain in the treatment.

2.5. Statistical Analysis. Statistical analysis was performed using SPSS for Windows, version 13.0 (SPSS, Inc., Chicago, IL, USA). Student's *t*-test was performed for comparisons of PRs/DRs between the groups. Differences were considered significant when *P* values were < 0.05. For actual cPR and cDR we used Kaplan-Meier method. For optimistic cPR and cDR we used modified Kaplan-Meier method which censors data for women who do not return for further IVF cycles to estimate the cPR/cDR of dropout patients with the same average chance of pregnancy/delivery as for those who returned for treatment.

3. Results

Our cohort included 395 women who attended 810 IVF cycles undergoing the first IVF treatment in year 2006 and all their subsequent repeated fresh or frozen-thawed cycles until the end of year 2012.

In their first IVF cycle women had the highest DRs regardless of the age; with each consecutive cycle the DRs were dropping. In the first cycle the DR was 28%, in the second 22%, and in the third 20%. In women under 38 years, a degree of DR was observed in each of six IVF cycles, while in women after 38 years there was no delivery after the second IVF cycle. DRs by women's age according to consecutive number of treatment cycle are presented in Table 1 (see Table 1).

In the longitudinal follow-up analysis, 395 women who started their first IVF cycle in year 2006 were taken into consideration. All first and subsequent fresh and corresponding frozen-thawed cycles of these women performed from year 2006 to the end of year 2012 were analysed. This analysis provided actual cPRs and cDRs for nearly 6 years of follow-up. The cPRs and cDRs, including fresh and thawed cycles, are shown in Figure 1.

The dropout of the patients was 20% (*n* = 77). In the group of women aged <38 years it was 19% (*n* = 60) and in the group of women aged ≥38 years it was 24% (*n* = 17).

The dropout patients are acknowledged as patients without a delivery in the first cycle and not returning for any further cycle. If those women were not included into the analysis, total cDR would have risen to 62% (66.5% in women aged <38 years versus 32.1% in women aged ≥38 years, resp.). Such exclusion was avoided by estimating the optimistic cDRs, statistically supplementing dropout patients with average actual cDR of the programme (see Figure 1).

Actual cPRs and cDRs were calculated according to women's age and ovarian response (see Table 2). In the group of low-responding women after the first IVF cycle there was statistically important difference in DR between women aged <38 years and women aged ≥38 years (0.3 versus 0.09; *P* = 0.018) as well as in actual cDR (0.50 versus 0.19) after available 6 IVF cycles (*P* = 0.003).

In the observed time period the average number of performed cycles per women was 1.91 in the group of women aged <38 years and 2.25 in the group of women aged ≥38 years.

We also calculated the optimistic cDR according to the women's age which assumes that women not repeating IVF cycles would have had the same chance to conceive as those women who maintain in the repeating system for 6 IVF cycles.

Among the women aged <38 years, the optimistic cDR after 6 IVF cycles was 83%. Among the women aged ≥38 years, the optimistic cDR after 6 IVF cycles was only 27%. The difference between optimistic and realistic cDR in younger women (<38 years) was 29% but in older women (≥38 years) was only 3% (Figure 2).

4. Discussion

The results of this study showed that in younger women (<38 years) the real cDR was 54% and optimistic cDR was 83%, respectively. In older women (≥38 years) the real cDR was 24% and optimistic cDR was 27%. These results enable us to report that prospects of the treatment in younger women, if they undergo all 6 available IVF cycles, are very high and quite comparable to the spontaneous conception. Even in older women it appears to be beneficent to repeat IVF cycles. This confirms the importance of repeated IVF cycles in assisted procreation and consequentially the importance of full coverage reimbursement IVF programmes.

In European countries, the IVF birth rate has been estimated to represent between 1% and 4.5% of all deliveries. In Slovenia birth rate reaches 4.5% of all deliveries; in the last European IVF Monitoring (EIM) report for the year 2009 it was the highest in Europe, besides Denmark, one of the leading European countries in the ART programme [1]. This can be achieved within fully reimbursed IVF systems which

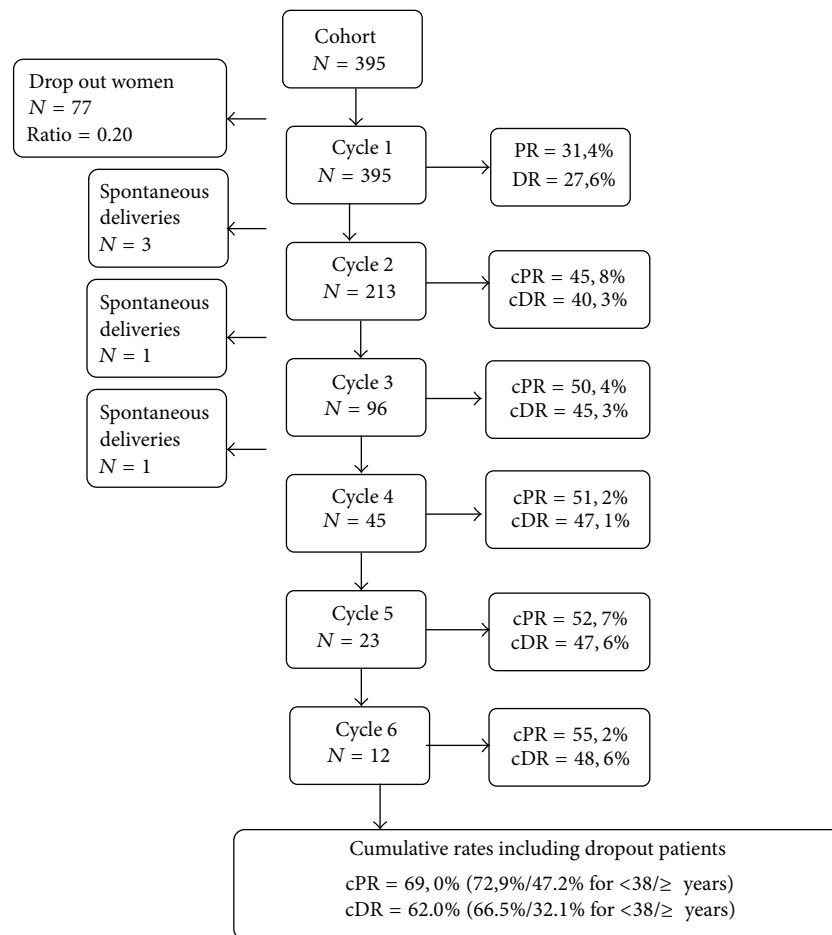


FIGURE 1: A flowchart of the total cohort of women who started the first IVF cycle in 2006 with subsequent repeated cycles including frozen-thawed cycles and the discontinuation rate (dropout patients). The middle rectangles represent six cycles and number of women participating in each of the cycle. The cPR and cDR are calculated for each cycle in the right rectangles. Dropout and spontaneous deliveries are presented in left rectangles. The actual cPR and cDR according to the age are presented in the bottom rectangle. Legend: cPR: cumulative pregnancy rate; cDR: cumulative delivery rate.

allow patients to repeat the IVF cycles and improve cDRs (as shown above). We consider such data to be important for the value and quality of the IVF programme in the country.

Recently, the usual annual cross-sectional analysis of PRs and DRs per started cycle and per embryo transfer was performed by the EIM which has collected the clinical data of most of European countries (IVF centres), respectively [1]. Despite being important, these data do not provide the information on the potential outcome and success of treatment for the individual couple or patient, since they do not show the overall success in repeating IVF cycles. Therefore the longitudinal analysis to provide the cDRs seems to be of greater importance.

Therefore we primarily reported the actual cDR in the IVF programme using longitudinal analysis. We were able to do this with accuracy, since there are only three IVF centres in Slovenia. We were able to gather data from all IVF centres in the country and thus avoid bias problems related to “moving patients” within centres. We believe that, so far, the present analysis may be the first one revealing data on the

IVF treatment in the whole country and not only data related to individual IVF centre [18–20].

Additionally, with the statistical optimistic assumption we have the opportunity to report to the couples/patients their optimal expectation, if they undergo all 6 cycles being available within the programme. Nevertheless, the figures on optimistic cDRs may be overestimated and must be interpreted with a caution [18–30]. Therefore both real actual values and optimistic estimations must be considered simultaneously.

The actual cDR for the whole cohort of patients was 49% while the optimistic estimate of the DR, which assumed that patients, who did not repeat IVF cycles, had the same chance of a delivery, reached 74%. This is in agreement with the Boston IVF centre achieving cDR between 51% and 72% performing conservative and optimistic calculation [27]. This comparison is feasible because of similar insurance policy for achieving the first delivery in both environments; in Massachusetts, the insurance benefits of up to six IVF cycles were offered to many patients [27].

TABLE 2: Pregnancy rates and delivery rates of first cycles started in year 2006 and actual cumulative pregnancy rates until year 2012.

			P value	Total
Age (years)	<38	≥38		
First cycle in 2006 (<i>n</i>)	324	71		395
PR (<i>n</i>)	0.33 (106)	0.25 (18)	0.208	0.31 (124)
DR (<i>n</i>)	0.30 (97)	0.17 (12)	0.013	0.28 (109)
Normal responders (<i>n</i>)	270	40		310
PR (<i>n</i>)	0.33 (88)	0.28 (11)	0.521	0.32 (99)
DR (<i>n</i>)	0.30 (81)	0.23 (9)	0.305	0.29 (90)
Low responders (<i>n</i>)	54	31		85
PR (<i>n</i>)	0.33 (18)	0.22 (7)	0.287	0.29 (25)
DR (<i>n</i>)	0.30 (16)	0.09 (3)	0.018	0.22 (19)
Cumulative pregnancy and delivery rates until the end of 2012				
Average number of cycles-total	1.91	2.25	0.053	1.97
Average number of cycles-normal/low responders	1.87/2.13	2.02/2.54		1.89/2.28
cPR (<i>n</i>)	0.58 (189)	0.35 (25)	<0.001	0.54 (214)
cDR (<i>n</i>)	0.54 (175)	0.24 (17)	<0.001	0.49 (192)
Normal responders (<i>n</i>)	270	40		310
cPR (<i>n</i>)	0.59 (158)	0.35 (14)	0.005	0.55 (172)
cDR (<i>n</i>)	0.55 (148)	0.28 (11)	0.001	0.51 (159)
Low responders (<i>n</i>)	54	31		85
cPR (<i>n</i>)	0.57 (31)	0.36 (11)	0.052	0.49 (42)
cDR (<i>n</i>)	0.50 (27)	0.19 (6)	0.003	0.39 (33)

Legend: PR: pregnancy rate; DR: delivery rate; cPR: cumulative pregnancy rate, and cDR: cumulative delivery rate.

Like several other investigators, we found that patients who did not return for further IVF cycles had worse chances to conceive than those who returned and continued with treatment [18, 19, 21, 25, 27].

It is known that fertility competence declines with increased female age in both the general population [18] as well as in subfertile women [31]. In our study, in younger women (<38 years) the repetition of IVF cycles resulted in a similar cDR in normally responding and in low-responding patients (54.8% and 50.0%, resp.). By estimating optimistic cDR which assumes that women who did not return for subsequent IVF cycles had the same chance of delivery as those who remained in the treatment, we reached the value of 83% cDR in younger women (<38 years). This result enables us to report to the younger women that their optimal expectation, if they repeat all 6 IVF cycles, comes very high and seems to be comparable to the spontaneous conception [18, 32].

On the other hand, the situation is different in older women (≥38 years). In normal-responding older women cDR was 28% and in low-responding women was 19%, which brings an average of 24%. All deliveries were achieved in the first or second IVF cycle. From the literature it is known that the overall live birth rate per started cycle for women who initiated an ART cycle at age 40 years or above is 9.7% and cumulative delivery rate is 28% [20]. It is interesting that 87% of women in this group who achieved delivery conceived a child within the first three IVF cycles [20]. This

is also in accordance with the results of our study; however we believe that larger number of older patients would have to be included in the study (sustaining the treatment after the third cycle) and meta-analysis should be performed. This may be achievable in cross-border studies. According to the data from the literature and our own data the age limit of 43 years is reliable, since assisted reproductive technology has a reasonable, beyond futile chance of success (>5% delivery rate) until the age of forty-three years [20].

If IVF treatment in Slovenia had not been fully reimbursed (6 cycles and additional 4 cycles after delivery), we would have not achieved such success in repeated IVF cycles. From the literature it is known that relatively large number of clinical pregnancies is achieved by repeated IVF attempts [33]. We strongly believe that, in the case of payable IVF cycles, these children would not have been conceived, since infertile patients usually do not sustain expensive IVF treatments [34]. Such situation was experienced in Germany after the German healthcare modernisation law had been implemented in year 2004, whereas the new legislation introduced the 50% copayment of the total ART treatment instead of the full reimbursement provided by the state. This led to a drastic reduction in ART treatment cycles per year, from 105,576 cycles in year 2003 to 61,950 cycles in year 2004 and 59,117 cycles in year 2005 [35], and showed that the lack of reimbursement can act as a barrier to people using ART and decrease a number of children born in the IVF programme [36, 37].

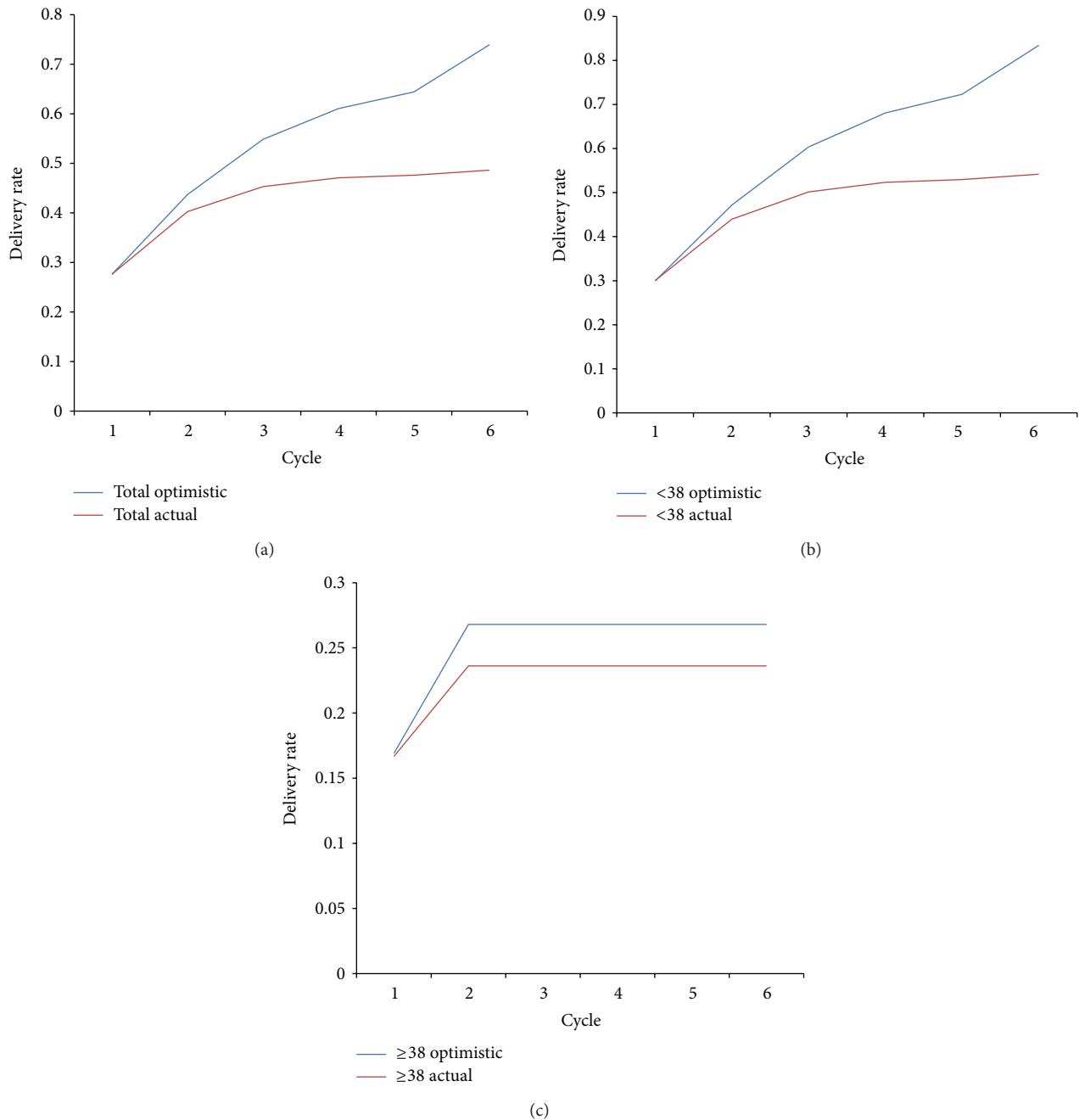


FIGURE 2: Actual and optimistic realistic cumulative delivery rate (cDR) in cohort (a), women aged <38 years (b), and women aged ≥38 years (c). Legend: cDR: cumulative delivery rate.

5. Conclusion

Our results show that by repeating IVF cycles actual cDR was 49% while optimistic (estimated) cDR was 74%. In younger women (<38 years), the actual cDR was 54% and optimistic cDR was 83%, while in older women (≥38 years) the actual cDR was 24% and optimistic cDR was 27%. Considering high cDRs in younger women, which are close to natural conception rates, we encourage early and sustained IVF treatment.

On the basis of overall reported actual and optimistic cDRs we may conclude that broad inclusion of patients (only limited by age) and full reimbursement of costs of IVF treatment by the state for 6 consecutive cycles and 4 consecutive cycles after a delivery are proven to be beneficent for the couples. No such cDRs would have been achieved if such system had not been established, since many patients do not repeat cycles if they are obliged to contribute financially to the treatment [35, 36]. Even the dropout which is not cost-related (difference between actual and optimistic DRs, 29%

in younger women (<38 years) and 3% in older women (≥ 38 years)) shows the importance of repeated cycles.

From the legal perspective, we may observe, however, that these elements have not been fully instrumentally implemented into the Slovenian legislation. Namely, Infertility Treatment and Procedures of Biomedically Assisted Procreation Act [7] covers only the basic principles of IVF treatment whereas the specifics (including age limit and number of cycles) are left to be governed by the regulation of Health Insurance Institute of Slovenia [4] which can be changed by the bodies of the institute under consent of the Ministry of Health if the rights are related to compulsory medical insurance. It has to be also noted that legislation [38] only provides 85% of reimbursement of the infertility treatment (assisted procreation) costs, while full reimbursement of costs is agreed within contractual relations between legal entities acting on behalf of IVF centres and Health Insurance Institute of Slovenia.

Since the right to assisted procreation is considered as a part of the constitutional Freedom of Choice in Childbearing [39, 40] and since only the legislation can determine the manners in which (constitutional) rights and freedoms are exercised [41] we believe that basic elements of the IVF system should be governed by the law. Such conclusion is also confirmed beyond legal argumentation with the findings of this paper on cDRs, showing that repeated reimbursed cycles are crucial in terms of exercising the right to (successful) IVF treatment. In terms of the equality in exercising constitutional Freedom of Choice in Childbearing the successful IVF treatment shall be considered as such only if it can deliver comparable results to the natural conception and thus help to eliminate differences between fertile individuals and those infertile individuals that can be helped by medical assistance in procreation.

Conflict of Interests

The authors declare that they have no financial or other conflict of interests.

Authors' Contribution

Urban Vrtacnik is responsible for paper drafting and critical discussion, Rok Devjak is responsible for data collection and analysis, and Eda Vrtacnik Bokal is responsible for paper study design, execution, paper drafting, and critical discussion.

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Clinical Study

Quantitative Elastography for Cervical Stiffness Assessment during Pregnancy

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Aim. Feasibility and reliability of tissue Doppler imaging-(TDI-) based elastography for cervical quantitative stiffness assessment during all three trimesters of pregnancy were evaluated. **Materials and Methods.** Prospective case-control study including seventy-four patients collected between the 12th and 42nd weeks of gestation. The tissue strain (TS) was measured by two independent operators as natural strain. Intra- and interoperator intraclass correlation coefficient (ICC) agreements were evaluated. **Results.** TS measurement was always feasible and exhibited a high performance in terms of reliability (intraoperator ICC-agreement = 0.93; interoperator ICC agreement = 0.89 and 0.93 for a single measurement and for the average of two measurements, resp.). Cervical TS showed also a significant correlation with gestational age, cervical length, and parity. **Conclusions.** TS measurement during pregnancy demonstrated high feasibility and reliability. Furthermore, TS significantly correlated with gestational age, cervical length, and parity.

1. Introduction

The process of cervical ripening precedes the active labor by several weeks and is defined as increased softening, effacement, and early cervical dilatation during pregnancy. *Digital cervical examination* remains the standard method for evaluating cervical ripening modifications. The Bishop score is a 10-point scoring system for assessing cervical dilation, effacement, consistency, and position, as well as fetal station [1]. Nonetheless, this approach is subjective and only semi-quantitative, and its specificity in patients with low scores is unsatisfactory [2]. Thus, an objective estimation of these parameters, inclusive cervical stiffness assessment, could be very important for the estimation of preterm delivery risk and labor induction success.

Elastography is a new imaging technique for the assessment of tissue stiffness by imaging the degree of tissue deformation (i.e., tissue strain, TS) [3, 4]. This tool could allow a quantitative and objective evaluation of the cervical

stiffness and thus potentially replace the current subjective and semiquantitative evaluation by palpation [5]. *Tissue Doppler imaging* (TDI) is a Doppler-based tool for the imaging and estimation of tissue strain (TS) using ultrasound. TDI allows for the tracking of tissue movement, while the TDI-Q (Q-quantification) software (Toshiba Medical Systems, Tokyo, Japan) facilitates an estimation of tissue stiffness based on the TS calculation [6]. Two previous preliminary studies have demonstrated the good reproducibility of the method and suggested its possible application for studying tissue elasticity during pregnancy. In these pilot studies, the standardization of the raw data acquisition techniques was addressed and demonstrated [7, 8]. Nonetheless, the feasibility and reliability of TS measurement in all three trimesters of pregnancy have not been investigated yet.

The aim of our study was to evaluate the feasibility and reliability of tissue Doppler imaging- (TDI-) based elastography for cervical quantitative stiffness assessment across all three trimesters of pregnancy.

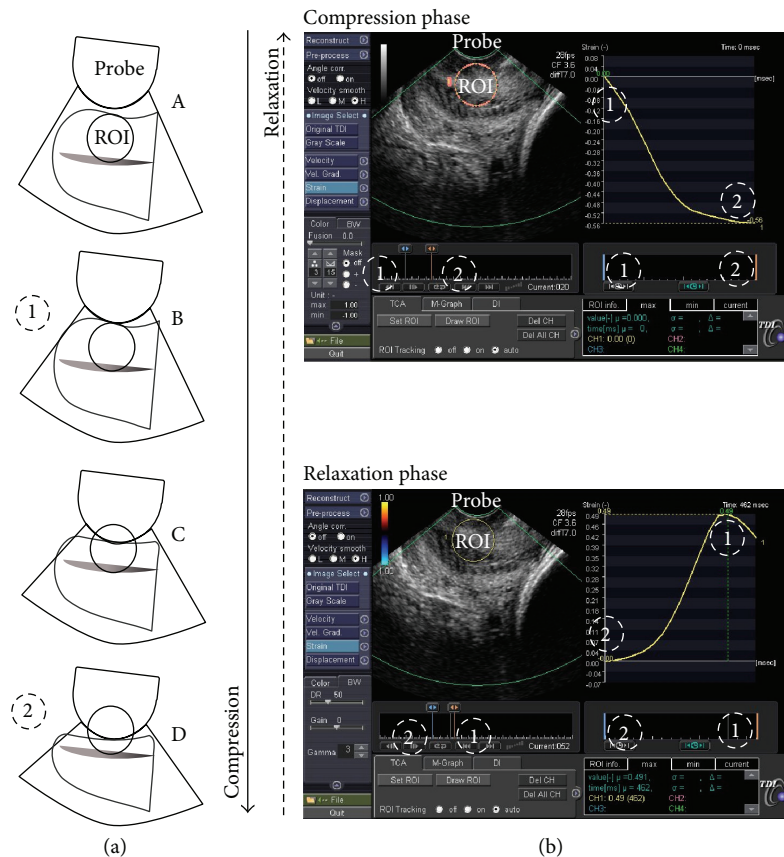


FIGURE 1: (a) Strain measurement process: the sequence of drawings (A, B, C, and D) shows one cycle of gentle compression and the subsequent relaxation of the anterior lip of the cervix through the vaginal probe, as previously described. (b) The process of TS calculation is displayed (above a compression and under a relaxation phase): a circular region of interest (ROI) is placed covering the whole thickness of the anterior cervical lip. The strain is then calculated during a compression phase (above: from a time of maximal relaxation, 1, to a time of maximal compression, 2), as well as during a relaxation phase (under: from a time of maximal compression, 2, to the time of the subsequent maximal relaxation, 1). The strain values are indicated as a function of the time during the movement (yellow line on the right).

2. Materials and Methods

2.1. Patients and Setting. The study was conducted prospectively. It was designed according to the Declaration of Helsinki and was approved by the local ethics board. Informed consent was obtained from all patients at the time of enrolment. The patients included 74 consecutive, unselected women, ranging from the 12th to the 42nd weeks of pregnancy recruited at the University Hospital of Rheine, Germany. The preexamination exclusion criteria included preterm membrane rupture or uterine contractions and a risk of preterm delivery. A minimal functional cervical length of approximately 15 mm (which conformed to the vaginal probe dimensions) was required to provide a sufficient cervical surface for an optimal examination. Indeed, a cervix shorter than the vaginal probe would not permit adequately compressing the cervix.

2.2. Strain Measurement. The process of TS measurement was subdivided into the following two steps: the first step consisted of the acquisition of raw data (*raw data set acquisition*) and the second step consisted of the analysis of the

acquired raw data, for the purpose of strain measurement (*TS calculation*). The process of strain measurement is described in Figure 1.

2.3. Raw Data Set Acquisition. Elastography was performed by a total of 2 gynaecologists. Each patient underwent a transvaginal, real-time elastography by the first operator, and two raw data sets were acquired (f^1 and f^2). Elastography was closely replicated by a second operator, and one additional raw data set was acquired (s^1). Real-time elastography was conducted using a 9 MHz vaginal probe and an Aplio XG ultrasound system (Toshiba Medical Systems, Tokyo, Japan). The procedure was executed as follows [7]: one to two cycles of the gentle compression-relaxation phases were exerted along the longitudinal axis of the cervix, avoiding the lateral and longitudinal dislocation of the tissues, until a maximal compression of its anterior portion was obtained (i.e., until no further shortening of the anteroposterior diameter could be observed and the posterior part of the cervical lip begins to be axially dislocated). In order to check the quality of the movements exerted, the transducer movements could be monitored in the real-time B-mode displayed on

the left panel of the screen, while the real-time elastography is contemporarily displayed on the right panel using the split-screen mode. Then, a five-second loop, including the last cycles of compression-relaxation, was acquired and stored in the machine as raw data. The raw data were acquired using TDI software (Toshiba Medical Systems, Tokyo, Japan).

2.4. Raw Data Set Analysis and TS Calculation. The TS calculations were performed offline using TDI-Q software. The procedure was executed as follows: the region of interest (ROI) tracking function was selected, and the natural strain preset was chosen with a derivative pitch value of 5 mm. A circular ROI was placed covering the whole thickness of the anterior cervical lip during the frame of the maximal tissue relaxation. Furthermore, the ROI should be placed along the axis of the compressing vaginal probe, in the middle part of the cervix. The TS values were then calculated during the relaxation phase (from the frame of maximal compression to the frame of the subsequent maximal relaxation). Strain values were measured considering the cycle with a larger compression-relaxation cervical tissue excursion. After manually selecting the cycle of interest with a cursor, the software automatically calculated the strain which occurred during the selected movement of compression (Figure 1). The strain calculation was executed by the first operator (F) both on its own acquired raw data set (Ff^1 and Ff^2) for the calculation of intraobserver variability and on the first raw data set acquired by the second operator (Fs^1) for the calculation of interoperator reliability. The TS calculation was then performed by the second operator (S) on its own acquired raw data set (Ss^1) and on the first raw data set acquired by the first operator (Sf^1) across the entire patient population, for the calculation of interoperator reliability.

2.5. Statistical Analysis. The data were analysed using R (version 2.14.1), and a significance level of $P < 0.05$ was considered to be significant. The data are presented as the median values and the interquartile ranges (IQR), the mean value and standard deviation, with prevalence and absolute values, or the reference values and 95% confidence intervals. First, we performed an analysis for each protocol to test the intraobserver and interobserver reliability of the raw data set acquisition and the TS calculation (per protocol). Then, we conducted additional tests to better assess the reliability of TS measurement (other analysis). For the reliability analysis of the raw data acquisition and TS calculation, we used the following tests: intraclass correlation coefficient (ICC), the mean of differences, and the difference between TS values/mean of TS values (percentage difference). The percentage difference is defined as the difference between two values divided by the average of the two values. It is shown as a percentage and we used it to show the amount of differences between two measurements. Moreover, we used the Bland-Altman plots of the average against the differences of the two measurements, and the limits of agreement were set as two standard deviations from the mean of the differences. We also plotted the 95% confidence intervals of the mean of differences to assess if the no difference line was inside

TABLE 1: Population features.

Gestational age at examination (weeks)	29.08 (± 8.93)
First quartile (12–20 weeks' gestation)	25.7% (19/74)
Second quartile (21–29 weeks' gestation)	23.0% (17/74)
Third quartile (30–37 weeks' gestation)	25.7% (19/74)
Fourth quartile (38–42 weeks' gestation)	25.7% (19/74)
Gravidity	2 (1–2)
Parity	1 (1–2)
Para = 0	24.3% (18/74)
Para ≥ 1	75.7% (56/74)
Cervical length (mm)	36.9 (± 10.17)
Number of fetuses	
One	97.3% (72/74)
Twins	2.7% (2/74)
Pregnancy outcomes	
Mode of delivery	
Ongoing pregnancy	41% (30/74)
Vaginal spontaneous delivery	38% (28/74)
Cesarean section	20% (15/74)
Operative delivery	1% (1/74)
Gestational age at birth (weeks)	38.8 (± 1.8)
Birth weight (grams)	3238.41 (± 641.05)

Description of the studied population. The values reported are mean (\pm standard deviation), median (interquartile range), or prevalence.

or outside of this range. We also used the following tests for the continuous variables: one way ANOVA, Kruskal-Wallis test, t -test, or Wilcoxon test. For the categorical variables, we used Chi-square or exact Fisher tests, where appropriate. To assess the correlations, we used locally weighted scatter-plot smoothing, least squares line, linear regression, and Pearson's test, where appropriate. Finally, we used Levene's test for the homogeneity of variances to assess the degree of variance of differences among the average measurements.

The STARD (Standards for Reporting of Diagnostic Accuracy) criteria for the accurate reporting of studies of diagnostic accuracy were considered [9].

3. Results

3.1. Population Description. The mean gestational age at the time of examination in the 74 women considered in the confirmatory phase (including the 17 women of trial phase) was 29.08 weeks (± 8.93). Of these, 41% had an ongoing pregnancy at the time of the statistical analysis of the study results, whereas 59% had already delivered, all at term of pregnancy and without pregnancy-associated complications. The characteristics of the study population are summarised in Table 1.

3.2. Reproducibility of Raw Data Set Acquisition and TS Calculation. In Table 2, we show the reproducibility of raw data acquisition and the TS calculation during the validation phase. We note that the reproducibility of the

TABLE 2: Reliability of tissue strain measurement.

	ICC consistency	ICC agreement	MD	SD	D/M of TS
Reliability of strain calculation					
Intraobserver (Ff^1/Ff^1)	0.97 (0.95/0.98)	0.97 (0.95/0.98)	-0.005 (-0.015/0.006)	0.047	7% (5–8%)
Interobserver (Ff^1/Sf^1)	0.98 (0.97/0.99)	0.98 (0.97/0.99)	-0.007 (-0.017/0.002)	0.041	5% (4–6%)
Interobserver (Fs^1/Ss^1)	0.99 (0.99/1.00)	0.99 (0.99/1.00)	-0.001 (-0.007/0.004)	0.023	3% (3–4%)
Interobserver (Ff^1/Sf^1 and Fs^1/Ss^1)	0.99 (0.98/0.99)	0.99 (0.98/0.99)	-0.004 (-0.010/0.001)	0.033	4% (3–5%)
Reliability of raw data acquirement					
Interobserver (Ff^1/Fs^1)	0.89 (0.84/0.93)	0.89 (0.82/0.93)	-0.029 (-0.050/-0.007)	0.092	12% (9–15%)
Interobserver (Sf^1/Ss^1)	0.91 (0.86/0.94)	0.91 (0.85/0.94)	-0.020 (-0.040/0.000)	0.084	10% (8–13%)
Interobserver (Ff^1/Fs^1 and Sf^1/Ss^1)	0.90 (0.86/0.93)	0.89 (0.85/0.92)	-0.024 (-0.038/-0.009)	0.089	11% (10–13%)
Reliability of raw data acquirement and strain calculation					
Intraobserver (Ff^1/Ff^2)	0.93 (0.89/0.96)	0.93 (0.90/0.96)	0.002 (-0.015/0.019)	0.072	10% (8–13%)
Interobserver (Ff^1/Ss^1)	0.90 (0.85/0.94)	0.89 (0.82/0.93)	-0.029 (-0.050/-0.008)	0.088	12% (9–14%)
Interobserver ($Ff^1/Ff^2/Ss^1$ —average of 2 raw data)	0.93 (0.89/0.96)	0.93 (0.89/0.96)	0.001 (-0.016/0.018)	0.072	10% (8–13%)

Reproducibility of tissue strain measurement, including raw data acquirement and tissue strain (TS) calculation. In brackets the 95% confidence interval is reported. MD: mean of differences; SD: standard deviation of MD; D/M of TS (= percentage difference): difference between TS values/mean of TS values (mean value and CI95).

TS calculation was high, with an ICC agreement ranging between 0.97 and 0.99. Even if we consider the Bland-Altman plots, most of the differences were within the region of agreement (\pm two standard deviations) and the average discrepancy between measures (bias: space between dotted zero line and mean of differences line) was inconsistent (Figures 2(a), 2(b), and 2(c) and Table 2). Therefore, we had observed an intra- and interobserver reproducibility of the TS calculation, as well as a high intraobserver reproducibility of the raw data acquisition and the TS calculation (Figure 2(d)). Across all the Bland-Altman plots (Figure 2), there were no significant trends to obtain larger or smaller differences between measurements, as the average increased ($P > 0.05$). Moreover, we tested the variability of the scatter around the bias line across the graph using Levene's test for homogeneity of variance, and the only relevant values are shown in Figures 2(f) and 2(b) (one measurement) and Figure 2(c) (mean of two raw data measurements), which showed significance of $P = 0.054$, $P < 0.05$, and $P = 0.399$, respectively. Furthermore, regarding the interobserver reproducibility of the raw data acquisition (Figures 2(e), 2(f), 2(b), and 2(c) and Table 2), we found the lowest ICC values (ranging between 0.89 and 0.91); however, these values were in the moderate to high agreement regions [10]. If we look at the Bland-Altman plots, we note the presence of a bias (indicated by the white space between the dotted zero line and the mean of the differences line) in all the plots that consider the interobserver variability of raw data acquisition or acquisition

and TS calculation (Figures 2(e), 2(f), and 2(b)). Bland-Altman plots the distance between the black line (the mean of differences) and the horizontal middle dotted zero line, and this space is defined as bias (= systematic error) (see Figures 2(e) and 2(f)). The presence of this bias raises the question of whether this average discrepancy between operators (i.e., the bias) is of clinical importance (i.e., if the intra- and interoperator variability of measurements are greater than the variability due to differences among gestational age groups). Unfortunately, we lacked the data to provide such an answer because the clinical usage of this test in the evaluation of cervix has not yet been assessed. Regardless, to evaluate the possible clinical relevance of this average discrepancy between the operators (i.e., the bias), we made some further considerations. First, in Figures 2(e), 2(f), and 2(b), the dotted zero line was out of the 95% confidence interval range of the mean of differences (gray band), while in the other plots, this was not the case. Moreover, in Figure 2(i), considering the mean of the two raw data acquisitions and the TS calculations of F for the comparison, along with the one raw data acquisition and TS calculation of S, the bias was corrected for and Levene's test became nonsignificant. Second, the mean cervical TS at 12–20 gestational weeks was 0.35 (± 0.07), at 21–29 gestational weeks was 0.41 (± 0.15), at 30–37 gestational weeks was 0.65 (± 0.12), and after 37 weeks was 0.73 (± 0.09 , $P < 0.05$). The difference between every two values of the above TS values in relation to the mean of every two considered values (percentage difference) had a

mean value of 43.17% ($\pm 24.00\%$) (CI95 27.05–59.29%), while the percentage difference due to the interobserver variability (D/M of TS, mean, and CI95) had values which ranged between 10 and 12% (Table 2).

3.3. Relevant Correlations of Cervical TS with Main Population Features. Finally, we found a significant correlation of the TS with gestational age and cervical length (Figure 3). As full-term pregnancy approached, the TS values increased, reflecting softer cervical tissue. Moreover, a shorter cervical length more frequently correlated with a high TS (again, indicating a softer cervix) (Figure 3). Overall, the pluripara women exhibited shorter and softer cervical properties, compared to the nullipara women ($P < 0.05$). Correlation between TS and cervical length and parity for each subcategory were not significant (except for TS in the 21st to 29th gestational age), probably due to the low number of patients considered in each subgroup (Table 3).

4. Discussion

Developing a new imaging technique for an objective assessment of tissue stiffness is potentially an important topic in obstetrics. This could be helpful for an objective and noninvasive description of the physiologic modification of the cervical stiffness occurring during pregnancy and for a better estimation of preterm delivery risk and labor induction success. Other authors tried to address this topic. Tekesin et al. proposed the quantitative ultrasonic tissue characterization (QUTC) tool, a software-based automated tissue gray-scale analysis process. Nonetheless, this tool is based only on a static B-mode picture of the cervix and the insufficient interobserver reliability limited its further development [11]. Recently Swiatkowska-Freund and Preis described a cervical elastography tool where the entity of tissue movements against the vaginal probe during pelvic arterial pulsation and breathings movement were represented on a colour map. Unfortunately, this colour-based Doppler analysis allows only semiquantitative evaluation of tissue movements on a scale from 0 to 4. Furthermore, clinical relevance of these findings has yet to be confirmed [12]. Molina et al. and Fruscalzo and Schmitz proposed a similar approach to that proposed by our group, demonstrating the reliability of quantitative elastography but failing to find the way to standardize the applied pressure needed for translating the tool to the clinical practice [13, 14]. Finally, Hernandez-Andrade et al. demonstrated how, also in a manually generated semiquantitative elastography, cervical tissue strain was related to the most important clinical characteristics of a nonselected population of pregnant women (in particular parity and cervical length). They showed also how cervical tissue strain was more strongly associated with cervical length than with gestational age. This could be explained by the fact that cervical softening, accompanied by cervical shortening, is not always, even if usually, directly related to the advancing gestation age [15]. Similarly, significant correlation between the cervical length and cervical elasticity was reported in their preliminary results by Fuchs et al. [16].

In this study, the natural strain was chosen to test for a universal setting for the TS measurement that could be applied throughout the whole gestational period. Indeed, the Lagrangian, but not the natural strain, failed to perform in the 3rd trimester, when the cervix is softer and the tissue deformation increases [8]. The strain can be calculated both as Lagrangian and natural strain. Whereas the Lagrangian strain describes the deformation (ϵ) of an object with its length $L(t)$ relative to its initial length $L(t_0)$ ($\epsilon L(t) = [L(t) - L(t_0)]/L(t_0)$), the natural strain is based on the temporal integration of the instantaneous deformation ($d\epsilon$) of the tissue ($d\epsilon N(t) = [L(t + dt) - L(t)]/L(t)$) [17]. In practice, for Lagrangian strain, we just need a start and an end dimension to calculate the strain while natural strain will be calculated over several measurement points. In general, Lagrangian strain is thought to be more accurate than natural strain when small deformations are measured, while the natural strain is thought to be more appropriate in the case of heterogeneous tissues or large tissue deformations (>10 – 15%). Indeed, the software is less accurate in the strain calculation than Lagrangian strain when following the ROI during large movements. While using natural strain calculation, the measured values are less dependent on the definition of the initial length L_0 [6]. As discussed in our previous studies [7, 8], in order to standardize the raw data set acquirement process, the compressing force was exerted until a maximal compression of its anterior portion was obtained and the cervical lip begins to be dislocated without further compression. Furthermore, in order to standardize the procedure of strain calculation, the ROI chosen was placed on the full thickness of the anterior cervical lip. Positioning the ROI on different levels of the target tissue (anterior and posterior cervical lip) introduces an important bias on the strain measurement. Indeed, different portions of the cervix are subjected to different forces depending on the distance from the compressing probe (due to the absorption of the force). Otherwise, examining the proximal and distal part of the cervix will imply exerting the compression movements not more perpendicularly to the cervix and introducing a shear strain component that actually cannot be calculated. Furthermore, the distal part of the cervix would slip away during the compression movements, and its lack of stability would compromise the principles of standardisation of the applied force. Finally, a minimal cervical length was required (we proposed a value of 15 mm, fitting the dimension of the vaginal probe) because the compression force should be exerted perpendicular to the longitudinal axis of the cervix. Indeed, it is intuitive that a very short cervix (shorter than the vaginal probe) does not permit adequately compressing the cervix by the probe [7, 8].

The results obtained using the proposed setting (5 mm DP during a relaxation phase) showed excellent results. A high reliability was shown when comparing two measurements of the same raw data set, both if they were calculated by the same operator and if they were calculated by another operator (indicating intra- and interobserver reliability for the TS calculation). These findings indicate that the process of TS calculation using the TDI-Q software has been properly standardised and appears to be stable.

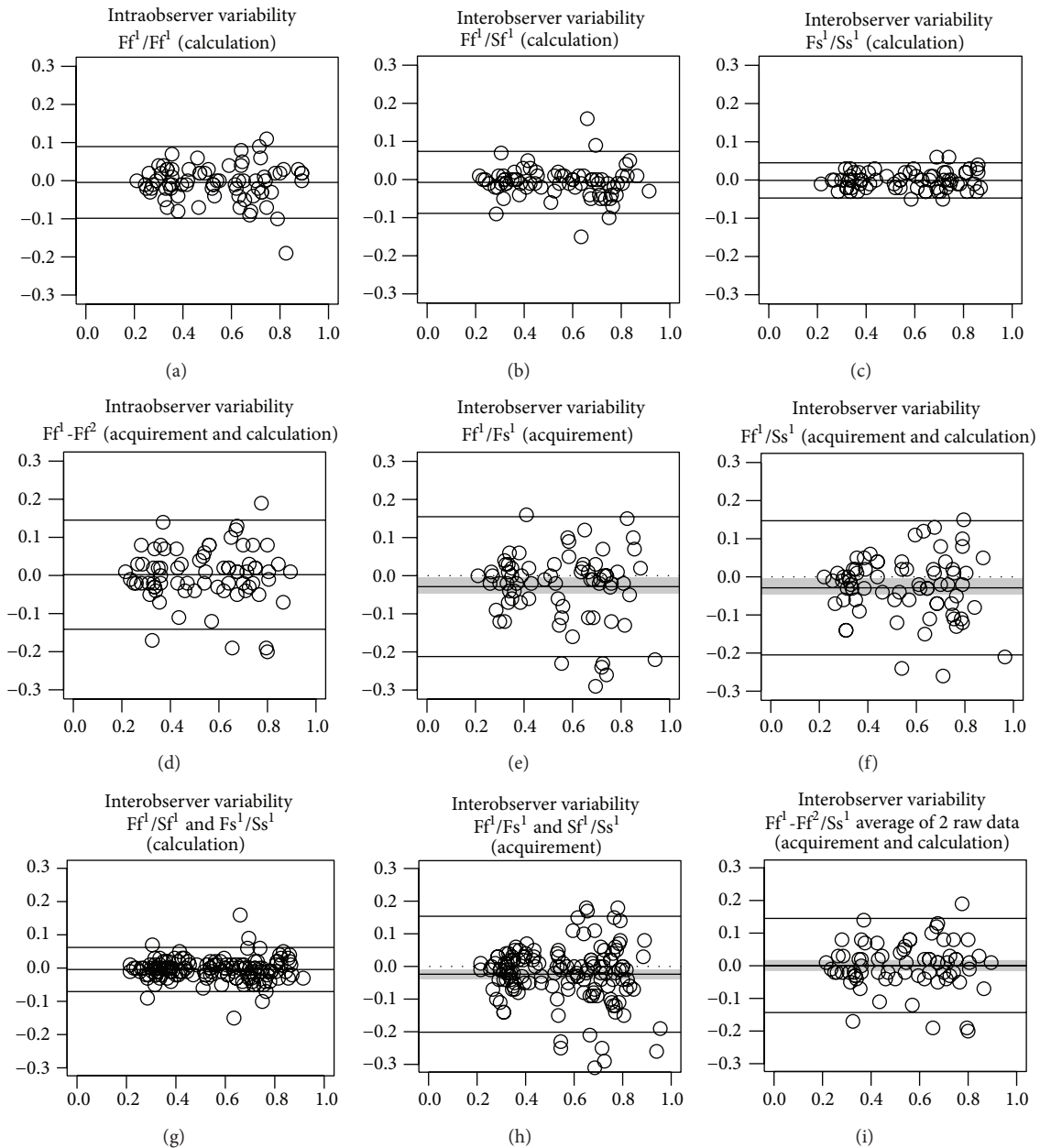


FIGURE 2: Reliability of tissue strain measurement. Plots (a), (b), (c), (d), (e), and (f) (per protocol analysis): the Bland-Altman plots demonstrate the degree of concordance between the pairs of cervical TSs. The region of agreement is included inside the two standard deviations interval from the mean of differences. The space included between the zero dotted lines and the mean of differences represents the bias. The gray band, where plotted, represents the 95% confidence interval of the mean of differences. F and S refer to the operator undergoing the TS calculation; f and s refer to the operator undergoing the raw data acquisition, where f^1 and s^1 and f^2 and s^2 refer, respectively, to the first and second raw data sets. (a) The intraobserver variability of the TS calculation of the same raw data (Ff^1/Ff^1) is shown. (b) The interobserver variability of the TS calculation of the same raw data (Ff^1/Sf^1) is shown. (c) The interobserver variability of the TS calculation of the same raw data (Fs^1/Ss^1) is shown. (d) The intraobserver variability of the raw data acquisition and calculation ($Ff^1 - Ff^2$) is shown. (e) The interobserver variability of the raw data acquisition with two raw data sets acquired by different investigators (f and s) and calculated by the same investigator is presented (Ff^1/Fs^1). (f) The interobserver variability of the raw data acquisition and calculation by the two investigators is indicated (Ff^1/Ss^1). Plots (g), (h), and (i) (other analysis): the Bland-Altman plots demonstrate the degree of concordance between the pairs of cervical TSs. (g) The interobserver variability of the TS calculation: in this plot, we analyse all data from Figures 2(b) and 2(c) (i.e., Ff^1/Sf^1 and Fs^1/Ss^1). (h) The interobserver variability of the raw data acquisition: the two raw data sets acquired by the two investigators (F and S) are measured by the same investigator (Ff^1/Fs^1 and Sf^1/Ss^1). (i) The interobserver variability of the raw data acquisition and calculation is shown ($Ff^1 - Ff^2/Ss^1$, yielding an average of the 2 raw data f^1 and f^2). In this analysis we took into consideration the average of the raw data measurements of investigator F versus the TS acquisition and measurement of one raw data set by investigator S.

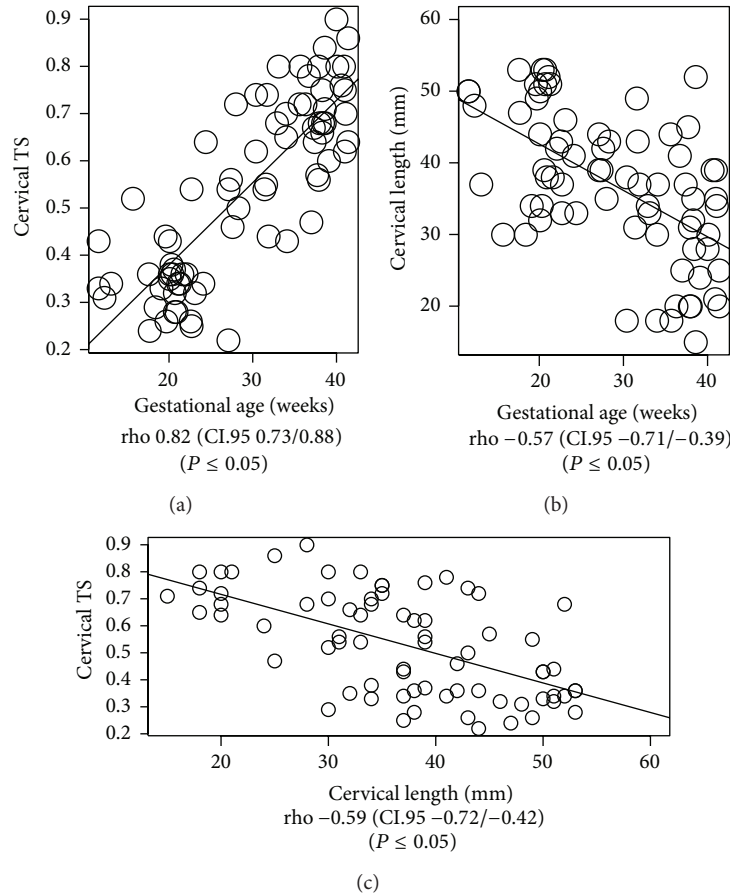


FIGURE 3: Tissue strain and patients' clinical features. The correlation of TS (average of two raw data set measurements) with the gestational age and cervical length (rho and P value refer to Pearson's test).

TABLE 3: Strain values and parity.

	N	Para = 0 ($n = 18$)	N	Para ≥ 1 ($n = 56$)	P
TS	18	0.37 (± 0.14)	56	0.59 (± 0.17)	<0.05
TS at 12–20 weeks' gestation	8	0.35 (± 0.09)	11	0.35 (± 0.05)	0.900
TS at 21–29 weeks' gestation	6	0.29 (± 0.05)	10	0.49 (± 0.14)	<0.05
TS at 30–37 weeks' gestation	3	0.58 (± 0.15)	16	0.66 (± 0.11)	0.455
TS at 38–42 weeks' gestation	0	NA	19	0.73 (± 0.09)	NA
Cervical length (mm)	18	41.47 (± 9.92)	56	35.37 (± 9.96)	<0.05
Cervical length at 12–20 weeks' gestation	8	43.12 (± 10.51)	11	44.55 (± 7.26)	0.748
Cervical length at 21–29 weeks' gestation	6	42.67 (± 5.35)	10	39.8 (± 5.73)	0.334
Cervical length at 30–37 weeks' gestation	3	34.67 (± 15.63)	16	32.93 (± 9.19)	0.868
Cervical length at 38–42 weeks' gestation	0	NA	19	29.33 (± 9.02)	NA

Tissue strain values related to parity and cervical length. Mean (\pm standard deviation).

Furthermore, a high reliability was shown for the TS measurements when comparing the three different raw data sets (indicating intra- and interobserver reliability for the whole process of TS measurement). The interobserver reliability was less consistent, due to the biases in raw data acquisition between the two operators. This difference could be overcome, for example, by repeating the measurement twice and calculating the mean TS, so that the eventual bias between

operators A and B will disappear. These results confirm the impression that the critical aspect of the TS measurement that accounts for measurement reliability depends upon the process of raw data acquisition.

Finally, our results were correlated with the clinical features of the study population. Specifically, the cervical TS seems to be related both to patient's gestational age and to her cervical length and parity. Therefore, unless the

relatively small number of patients used here does not allow for generalisations, these results indicate the potential clinical applicability of the TDI and TDI-Q tools for the quantitative measurement of TS.

4.1. Limitations of the Study. A major limitation of the proposed tool is the dependency of TS on the applied force, during the movement of the cervical compression [14, 18]. However, standardised conditions for the raw data acquisition (maximal exertion of compression and care in avoiding the dislocation of the cervix) can optimise the TDI-based TS measurements to enhance feasibility and reliability. The problems of the manual compression and unknown value of the applied force implicate the impossibility to calculate the absolute value of the elastic module of the cervix but allowed an estimation of the tissue stiffness. Recently a manual method for cervical stiffness evaluation was proposed, based on the calculation of the anteroposterior cervical diameter anteroposterior 10 cervical diameter measured before (AP) and after (AP') application of pressure on the cervix using the transvaginal probe. The author describes an excellent intra- and interobserver correlation, as well as a better prediction of preterm delivery compared to the cervical length measurement [19]. Thus, even if the technique of Parra measures the strain as Lagrangian strain while in our protocol the measure was made using the natural strain, the mechanism used for strain measurement remains the same. Nonetheless, comparison among different studies should be done with caution. Indeed, for a small deformation the natural and Lagrangian strains are similar, as the Lagrangian and natural strains are interrelated by a fixed mathematical formula: $(\epsilon N(t) = \ln(1 + \epsilon L(t)))$, but for a larger deformation (as induced in this study), the natural strain will always be greater [17].

The measurement of the stiffness in very short cervixes can be limited, due to the impossibility to direct the compressing force perpendicularly to the cervical tissue. However, assessing the risk of preterm delivery in an asymptomatic patient will be probably more interesting than in patients with a very ripened cervix, which are already known to be at great increased risk for preterm delivery.

Furthermore, in order to reduce the number of measurements to be performed, the feasibility and reliability study was first conducted testing some of these combinations among a restricted group of patients by a single operator and then tested among the whole study population. Thus, even if results demonstrated the high reliability of this tool using the chosen preset, this does not exclude that other settings could work even better than this. Further studies considering this topic comparing different settings and ROIs positioned in different regions of the cervix are currently ongoing. The clinical usefulness of this diagnostic tool should be now tested in large prospective clinical settings, even if preliminary results appear to be promising [20].

5. Conclusions

Under standardised conditions for the acquisition of raw data and strain calculation, the TDI-based cervical strain

measurements obtained during pregnancy are feasible and show high intra- and interoperator reliability. The strain measurements obtained during the relaxation frame using a DP of 5 mm proved to be a well-performing TDI-Q setting for the cervical TS measurement. An objective and quantitative estimation of cervical stiffness during pregnancy could be very important for the estimation of preterm delivery risk and could improve assessment of cervical ripening at term to select patients for successful labor induction. The clinical usefulness of this diagnostic tool should be now tested in large prospective clinical settings.

Ethical Approval

The paper was approved by Ethics Committee of the local College of Surgeons and the medical Faculty at the University of Münster (Reference number: 2010-256-f-S).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

A. Fruscalzo contributed to conception, planning, performing the experiments, and writing the paper; A. P. Londero contributed to statistical analysis and cowriting the paper; C. Fröhlich contributed to performing the experiments; U. Möllmann contributed to performing the experiments; and R. Schmitz contributed to performing the experiments and cowriting the paper.

Disclosure

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Research Article

Superoxide Dismutase: A Predicting Factor for Boar Semen Characteristics for Short-Term Preservation

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Superoxide dismutase (SOD), total antioxidant capacity (TAC), and thiobarbituric acid reactive substances (TBARS) in seminal plasma were evaluated on the basis of receiver operating characteristics (ROC) analysis as predictors for distinguishing satisfactory from unsatisfactory boar semen samples after storage. SOD on day 0 correlated significantly with progressive motility ($r = -0.686$; $P < 0.05$) and viability ($r = -0.513$; $P < 0.05$) after storage; TBARS correlated only with motility ($r = -0.480$; $P < 0.05$). Semen samples that, after 3 days of storage, fulfilled all criteria for semen characteristics (viability $> 85\%$, motility $> 70\%$, progressive motility $> 25\%$, and normal morphology $> 50\%$) had significantly lower SOD levels on the day 0 than those with at least one criterion not fulfilled ($P < 0.05$) following storage. SOD levels of less than 1.05 U/mL predicted with 87.5% accuracy that fresh semen will suit the requirements for satisfactory semen characteristics after storage, while semen with SOD levels higher than 1.05 U/mL will not fulfill with 100% accuracy at least one semen characteristic after storage. These results support the proposal that SOD in fresh boar semen can be used as a predictor of semen quality after storage.

1. Introduction

Evaluating the fertility of sperm is economically important in breeding management. The modern boar industry worldwide is based on the use of artificial insemination (AI) of sows with mostly cooled semen. Semen is stored at 15–20°C for 1 to 5 days in the liquid state after dilution in an appropriate extender [1]. However, after short-term liquid storage, the quality of boar spermatozoa is diminished [2]. Additionally, differences between individuals in boar sperm longevity and survivability are well known [3].

The true index of fertility is the pregnancy and farrowing rate; however, both are expensive, time consuming, and influenced by factors extrinsic to the boar, such as sow quality and breeding management. While poor semen quality is a good indicator of reduced fertility, good semen quality (in terms of concentration, motility, and morphological normality) is not necessarily a warrant of acceptable fertility and long lasting semen viability [4]. Moreover, despite having

normal or comparable sperm parameters in fresh semen, large differences have been noted in sperm parameters after short-term storage [5]. There is, therefore, a need for new sperm function parameter that would relate better to semen characteristics after storage and fertility.

Increasing evidences suggest that generation of reactive oxygen species (ROS) occurs during preservation of sperm [6]. Excessive ROS generation is detrimental to boar sperm cells and is associated with time-dependent decrease in motility, viability, and membrane permeability of spermatozoa during storage [7]. High sensitivity of boar semen to oxidative stress is due to the high content of unsaturated fatty acids in plasma membrane phospholipids and the relatively low antioxidant capacity of boar seminal plasma [8]. The mechanisms by which ROS disrupt the sperm functions are believed to involve the peroxidation of the polyunsaturated fatty acids present in the sperm plasma membrane [9].

Porcine seminal plasma is endowed with low molecular nonenzymatic and enzymatic defense mechanisms that can

protect against ROS [10], with high quantities of superoxide dismutase (SOD) [11]. SOD protects spermatozoa by catalysing the dismutation of superoxide anions to hydrogen peroxide and oxygen, thereby protecting mature spermatozoa against excessive superoxide anion accumulation [9]. Owing to the high ability of boar semen for superoxide dismutation and lack of catalase-like activity in boar spermatozoa or seminal plasma [11] hydrogen peroxide is suggested to be the primary source of oxidative damage in boar sperm [12]. Interestingly, by cooling boar semen down to 5°C intracellular levels of $\cdot\text{O}_2$ and H_2O_2 in sperm decrease [13]. As well, basal intracellular ROS formation is low in viable boar sperm of fresh and frozen-thawed semen [12].

The aim of our study was to determine whether oxidative stress biomarkers SOD, TAC, and TBARS in fresh semen could be helpful in predicting the quality of boar semen following short-term storage.

2. Materials and Methods

2.1. Semen Collection and Evaluation. Seventeen ejaculates were collected in spring from eight mature, healthy boars of various breeds (3 Slovenian landrace, 2 Slovenian large white, 2 Pietrain, and 1 Hibride line (54)) aging 12 to 24 months. All boars were fed commercial food (pellets) for AI boars and were housed in individual pens equipped with nipple drinkers to the European Commission Directive for Pig Welfare. Semen samples were collected routinely at the local AI centre (Ptuj, Slovenia) by the gloved-hand technique, using a clean semen collecting flask that filters out gel, dust, and bristles. Immediately after collection, semen was diluted 1:2 in Beltsville Thawing Solution (BTS, Truadeco, The Netherlands). While being analyzed, semen was kept at room temperature ($20^\circ\text{C} \pm 1^\circ\text{C}$) for one hour in order to decrease chilling injury [14]. Prior to sperm motility analysis, 1 mL of diluted semen sample was incubated at 37°C for 8 minutes. A computer assisted sperm analyzer (Hamilton Thorne IVOS 10.2; Hamilton Thorne Research, MA, USA) with a Makler counting chamber (Sefi Medical Instruments, Israel) was used to evaluate motility and progressive motility. Sperm concentration was measured with a photometer (Photometer SDM 5, Minitüb, Germany) [15] while sperm viability was assessed using Hoechst staining [16]. The morphology of 200 spermatozoa was assessed in diluted semen samples after fixing in Giemsa stain [17].

Semen samples were then stored for 3 days in closed plastic containers in a thermal box. Temperature was controlled by calibrated thermometer and was kept at $16\text{--}17^\circ\text{C}$. Semen samples were constantly gently agitated in order to preserve the quality of stored semen [18]. The sperm characteristics of liquid-stored semen samples were evaluated after 3 days (72 h) of semen preservation, in the same way as that described above.

All samples were assessed in duplicates.

2.2. Preparation of Seminal Plasma and Analysis of Oxidative Stress Biomarkers. Extended semen was centrifuged at $818 \times g$ for 10 minutes at room temperature. The supernatant was

removed and further centrifuged at $13,000 \times g$ for 15 min at 4°C to separate seminal plasma, which was then aliquoted and frozen at -80°C until assayed for TBARS, TAC, and SOD.

Semen samples were assayed for TAC by an automated biochemistry analyzer RX Daytona (Randox, Crumlin, UK), using a commercially available Total Antioxidant Status (TAS) kit (Randox, Crumlin, UK) that is based on the original method of Miller et al. [19]. The results are expressed as $\mu\text{mol/L}$ of Trolox equivalents.

SOD activity (U/mL) was determined spectrophotometrically with an automated biochemical analyser RX Daytona (Randox, Crumlin, UK), using the Ransod kit (Randox, Crumlin, UK) that is based on the original method of McCord and Fridovich [20].

Lipid peroxidation was measured by using the thiobarbituric acid (TBA) reaction for malondialdehyde (MDA) (TBARS assay Kit, Cayman Chemical Company). $100 \mu\text{L}$ of seminal plasma from each sample was mixed with 2 mL of the TBA-TCA reagent (15%, w/v TCA; 0.375%, w/v TBA and 0.25 N HCl) and incubated in a boiling water bath for 60 min and subsequently cooled in an ice bath for 10 minutes. After cooling, the suspension was centrifuged at $1600 \times g$ and 4°C for 10 min. The supernatant was then separated, and absorbance was measured at 532 nm at room temperature over a period of 30 min after separation of supernatant. The assay was conducted in duplicate. The amount of TBARS produced ($\mu\text{mol/L}$) was quantified against a standard curve created using MDA as standard (Tecan, Safir 2).

2.3. Statistical Analysis

2.3.1. Correlation Analysis. Using Kolmogorov-Smirnov test, it was confirmed that data varies significantly from the pattern expected if the data was drawn from a population with a normal distribution; therefore, nonparametric tests were used in further evaluation.

Spearman rank correlation coefficient was used to determine the relationship between semen parameters on days 0 and 3. Statistical comparison of the results obtained on days 0 and 3 for each semen parameter was performed with the Mann-Whitney *U*-test. Statistical analyses were performed using Sigma Stat 3.5 (SYSTAT Software Inc., Illinois, USA). $P < 0.05$ was considered as significant.

2.3.2. Diagnostic Evaluation. Diagnostic evaluation was performed to determine whether the boar semen quality after 3 days of storage could be predicted from oxidative stress markers values measured on the day of semen collection. Semen samples were divided into satisfactory (SAT) and unsatisfactory (UNSAT) groups according to individual semen parameter after 3 days of storage. Criteria for SAT semen samples were viability: $>85\%$, motility: $>70\%$, progressive motility: $>25\%$, and normal morphology: $>50\%$.

Samples were further categorized based on the number of satisfactory parameters that each individual semen sample achieved. Samples with all four parameters determined to be "satisfactory" were placed in Group 1; all the other samples were included in Group 2.

TABLE 1: Summary of sperm parameters in AI dose ($n = 17$).

	Day 0		Day 3	
	Mean \pm SD	Range (minimum–maximum)	Mean \pm SD	Range (minimum–maximum)
Sperm concentration (million/mL)	279.0 \pm 68.1	154.8–382.2	279.0 \pm 68.1	154.8–382.2
Motility (%)	81.8 \pm 8.2	67.0–94.3	68.9 \pm 15.5	25.7–89.3
Progressive motility (%)	39.9 \pm 5.2	30.0–46.7	26.3 \pm 8.1	13.0–43.0
Morphologically normal form (%)	73.1 \pm 9.6	53.2–84.7	55.8 \pm 9.4	39.2–69.0
Viability (%)	93.2 \pm 4.1	79.0–97.0	87.8 \pm 4.4	76.0–93.5
Capacitated + acrosomal reacted (%)	13.1 \pm 8.8	4.5–45.5	37.0 \pm 11.4	22.5–66.5
TAC (μ mol/L)	808.8 \pm 185.0	395.0–1140.0	770.9 \pm 194.5	320.0–1100.0
TBARS (μ mol/L)	39.9 \pm 5.4	30.8–48.7	39.0 \pm 4.7	32.7–47.1
SOD (U/mL)	1.3 \pm 0.7	0.5–2.9	2.7 \pm 0.9	1.6–4.4

AI: artificial insemination; TAC: total antioxidant capacity; SOD: superoxide dismutase; TBARS: thiobarbituric acid reactive substances.

On day 0, samples were tested positive or negative according to SOD and TBARS. A positive test result (T+) was recorded when SOD or TBARS in seminal plasma was above the cut-off value. A negative test result (T-) was recorded when SOD or TBARS in seminal plasma was below the cut-off value. Together with the classification to SAT and UNSAT groups, four categories of results were obtained: true positive (TP), true negative (TN), false positive (FP), and false negative (FN). Diagnostic parameters, sensitivity, specificity, and positive and negative predictive values were calculated as described [21]. Sensitivity is indicated by the percentage of semen samples identified by the fresh oxidative stress semen parameter being unsatisfactory after 3 days of liquid storage. Specificity is a percentage of satisfactory samples after 3 days; samples were tested negative by fresh oxidative stress semen parameter. A positive predictive value (PPV) is the percentage of samples with a positive test result actually unsatisfactory after 3 days of storage. The negative predictive value (NPV) is the percentage of samples with a negative test result that are actually satisfactory after storage.

Receiver operating characteristic (ROC) curves were performed to determine the overall discriminating power of each semen variable. ROC curves plotted sensitivity versus 1-specificity for the complete range of cut-off points. All possible combinations of sensitivity and specificity that can be achieved by changing the breaking point are summarized by a single parameter, that is, the area under the ROC curve (AUC). A diagonal line in a ROC plot corresponds to a test that is positive or negative just by chance [22]. On the basis of the AUC for each semen parameter, we can determine whether the information is helpful in discriminating semen quality outcome after 3 days of storage or not. Helpful information about semen quality on day 3 is found when AUC close to 1 is observed together with statistical significance ($P < 0.05$).

ROC analysis was used to calculate the elective breaking point or cut-off value for oxidative stress markers in relation to semen quality after 3 days of liquid storage. Cut-off values for oxidative stress markers that can differentiate satisfactory from unsatisfactory semen samples after 3 days

of storage were chosen to maximise the sum of sensitivity and specificity.

ROC analysis was performed using Analyse-it for Microsoft Excel (version 1.71) (Analyse-it Software, Ltd. <http://www.analyse-it.com/>, 2009).

3. Results

3.1. Sperm Characteristics on Days 0 and 3. Semen parameters were measured for fresh semen samples (day 0) and for semen samples after 3 days of liquid storage (day 3) (Table 1). All basic semen parameters differed significantly after 3 days of storage ($P < 0.05$). The concentration of spermatozoa did not change.

3.2. Correlation between Oxidative Stress Markers and Semen Parameters in Fresh and Stored Semen. Spearman correlation coefficients between oxidative stress markers (TAC, SOD, and TBARS) on day 0 and all semen parameters after 3 days of storage are shown in Table 2.

SOD in seminal plasma on day 0 showed a significant correlation with progressive motility ($r = -0.686$; $P = 0.002$) and viable spermatozoa ($r = -0.513$; $P = 0.035$) after 3 days of storage. A negative correlation near the level of significance was observed between SOD on day 0 and morphologically normal spermatozoa on day 3 ($r = -0.423$; $P = 0.087$). A negative correlation between TBARS on day 0 and motility on day 3 was also observed ($r = -0.480$; $P = 0.054$), whereas TAC on day 0 did not show any significant correlation with semen characteristics on day 3.

3.3. Correlation between Oxidative Stress Markers in Fresh and Stored Semen. Correlations between oxidative stress markers on days 0 and 3 were also seen. TAC in fresh semen correlated strongly with TAC and TBARS in stored semen ($r = 0.918$, $P < 0.001$ and $r = -0.473$, $P = 0.054$, resp.). TBARS on day 0 and day 3 showed negative correlation ($r = -0.463$) but it was not statistically significant ($P = 0.059$). No significant

TABLE 2: Correlations (*r* value (*P*)) between oxidative stress markers in boar semen on day 0 and semen parameters after 3 days of storage.

Semen parameters and oxidative stress markers—day 3	Oxidative stress markers—day 0		
	TAC	TBARS	SOD
Motility	−0.135 (0.598)	−0.480* (0.049)	−0.187 (0.466)
Progressive motility	−0.120 (0.639)	0.123 (0.632)	−0.686* (0.002)
Morphologically normal form	−0.365 (0.145)	0.020 (0.936)	−0.423^a (0.087)
Viability	0.154 (0.546)	−0.309 (0.222)	−0.513* (0.035)
Capacitated + acrosomal reacted	0.282 (0.266)	0.135 (0.598)	−0.346 (0.169)
TAC	0.918** (<0.001)	−0.473^a (0.054)	0.223 (0.382)
TBARS	0.220 (0.387)	−0.463^a (0.059)	−0.186 (0.466)
SOD	−0.192 (0.454)	0.250 (0.326)	0.407 (0.107)

TAC: total antioxidant capacity; SOD: superoxide dismutase; TBARS: thiobarbituric acid reactive substances; *P*: statistical significance (**P* < 0.05; ***P* < 0.001); ^anear statistical significance.

TABLE 3: Cut-off values (breaking points) and diagnostic parameters of semen variables on day 0 and on day 3.

Variable—day 3	Breaking point	AUC (<i>P</i>)	Sens. (%)	Spec. (%)	PPV (%)	NPV (%)
SOD (U/mL)—day 0						
Progressive motility	1.22	0.86 (0.0003)	87.5	88.9	87.5	88.9
Mor. normal form	1.26	0.70 (0.0697)	80.0	75.0	57.1	90.0
Viability	1.26	0.85 (0.0002)	100	76.9	57.1	100
Group 1/Group 2	1.05	0.97 (<0.0001)	90.0	100	100	87.5
TBARS (μmol/L)—day 0						
Motility	40.73	0.68 (0.0888)	60.0	66.7	42.9	80.0

AUC (ROC): area under the curve (receiver operating characteristics); *P*: statistical significance; Sens.: sensitivity; Spec.: specificity; PPV: positive predictive value; NPV: negative predictive value.

correlation was found between SOD on day 0 and oxidative stress markers in stored seminal plasma (*P* > 0.05).

3.4. Diagnostic Evaluation. Cut-off values (breaking points) and diagnostic parameters of oxidative stress markers on day 0 are presented in Table 3.

Regarding each individual parameter, SOD in fresh semen provided relevant information about progressive motility and viability after storage (AUC 0.86 and 0.85, resp.; *P* < 0.05). Optimal cut-off values of SOD for distinguishing stored semen according to progressive motility and viability were similar (1.22 and 1.26 U/mL, resp.).

SOD in fresh semen showed the highest predictive value for progressive motility after semen storage; 87.5% of semen samples had less than 25% progressive motility after 3 days of storage, whereas only 57.1% (PPV) of semen samples according to viability were actually unsatisfactory (less than 85% viable spermatozoa) after 3 days of storage. The NPV was high; 88.9% of semen samples with a concentration of SOD less than 1.22 had a satisfactory progressive motility above 25% after storage and 100% of semen samples with a concentration of SOD less than 1.26 U/mL had more than 85% viable spermatozoa after storage.

On the basis of lower AUC (AUC 0.70, *P* = 0.070), SOD provided less useful information in revealing semen quality outcome based on normal morphology.

Semen samples classified in Group 1 fulfilled all criteria for satisfactory semen characteristics after storage, whereas

semen samples in Group 2 had at least one unsatisfactory semen characteristic after storage. Based on ROC analysis, the threshold point of SOD on day 0 was 1.05 U/mL (Table 3). Sensitivity indicated that the concentration of SOD in fresh seminal plasma was less than 1.05 U/mL in 90% of the semen samples in Group 1, whereas 100% of the semen samples in Group 2 had ≥1.05 U/mL SOD in the seminal plasma. Predictive values were high; 100% of all semen samples with SOD ≥ 1.05 U/mL had at least one unsatisfactory semen characteristic (Group 2) after storage. Samples with concentrations of SOD in fresh seminal plasma of less than 1.05 U/mL, however, maintained 87.5% (NPV) accuracy of all criteria for satisfactory semen after storage (Table 3). The ROC curve for SOD on day 0 for distinguishing Group 1 from Group 2 is shown in Figure 1.

On the basis of AUC, TBARS provided less helpful information in discriminating semen quality outcome on the basis of motility (AUC 0.68; *P* = 0.089) (Table 3).

4. Discussion

Our study documented that SOD activity in fresh seminal plasma is a valuable indicator of porcine semen quality following liquid storage for 3 days. Current measures of semen quality are not always indicative of semen quality after storage [5] and do not predict accurately the ability of short-term stored spermatozoa to fertilize [21]. New markers of sperm function that would enable better prediction of

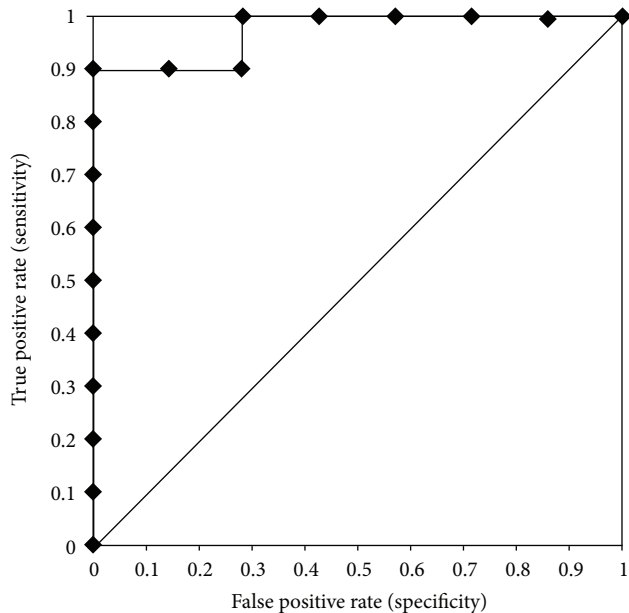


FIGURE 1: Receiver operating characteristics (ROC curve) for SOD on day 0 according to Group 1 and Group 2.

fertilizing ability in boars have been sought [21, 23]. In this study, we have evaluated the possibility of using oxidative stress markers in fresh seminal plasma as predictors of the quality of boar semen submitted to short-term storage.

Interestingly, TBARS levels during storage in our study did not change significantly. In previous studies, contradictory reports are found concerning TBARS levels after liquid storage; Boonsorn et al. found even decreased levels of TBARS [24], whereas Kumaresan et al. reported increased TBARS levels [5]. These contradictory results from different studies in boars are difficult to explain. Even though TBARS assay is a well-establish method for screening and monitoring lipid peroxidation, the specificity of TBARS is questionable, because any sugar can yield a pink coloured product [25]. This could be the cause of contradictory results in different studies. Therefore, other methods for estimating lipid peroxidation like HPLC or estimation of isoprostanes, which are more specific, should be used [25].

However, there was a significant negative correlation in our study between levels of TBARS in fresh semen and semen motility following storage ($r = -0.480$; $P < 0.05$). Higher levels of TBARS in fresh semen could indicate that spermatozoa were under increased oxidative stress, which lead to a reduced motility after storage. This significant negative correlation between TBARS and semen motility could support other authors' suggestion that sperm motility is a sensitive indicator of oxidative stress and probably one of the first parameters affected [26]. However, despite the observed correlation noted above, based on ROC analysis, TBARS was not a valid marker for predicting boar semen quality after storage.

Total antioxidant capacity of seminal plasma was reduced during liquid storage which is in agreement with a study

by Brzezinska-Slebodzinska et al. [8]. Am-In et al. reported that low TAC in seminal plasma is associated with lower storability of boar semen [27]. In the same study a significant negative correlation between TAC and percentage of normal sperm morphology was noted [27]. In our study levels of TAC in fresh semen samples did not correlate significantly with semen parameters after storage. These contradictory results could be due to experimental differences, including low numbers of ejaculates included in studies. Moreover, large numbers of sperm per dose could compensate for any fertility factor and mask any relationship with sperm quality [1]. Preselection of ejaculates by motility under commercial conditions tends to lead to lower variability in semen parameters, which could be manifested in stronger correlations than in the absence of preselection [28].

SOD activity in boar seminal plasma increased after three days of storage. Although SOD is a major antioxidant enzyme of boar seminal plasma [10], high levels of SOD activity are also found in boar spermatozoa [29]. Increased activity of SOD in seminal plasma could be related to the leakage of intracellular enzyme. Similar results are reported in bulls [30] and fowls [31], where SOD activity during storage decreases in spermatozoa but increases in seminal plasma. It was recently found that cooling boar semen from 15°C to 5°C may be primarily responsible for destabilization of sperm membranes (evaluated with 6-CFDA/PI), even though there was a decrease in intracellular levels of O_2 and H_2O_2 [13]. Therefore, it could well be that increased SOD activity in seminal plasma in our study resulted from the leakage of intracellular enzyme from spermatozoa due to destabilization of sperm membranes as a result of liquid storage, without any concurrent detectable lipid peroxidation measured by TBARS. Further studies that would include measurement of membrane stability are needed to confirm the prediction mentioned above.

We have shown here that SOD in boar seminal plasma on day 0 correlates significantly with progressive motility ($r = -0.686$; $P < 0.001$) and viability ($r = -0.513$; $P < 0.05$) measured on the third day of storage. A negative correlation with levels of morphologically normal spermatozoa was near the level of significance ($r = -0.423$; $P = 0.059$). The role of SOD as a predictor of spermatozoa lifespan has already been suggested in humans, where a good linear correlation between the rate of spontaneous lipid peroxidation and SOD activity was observed [32]. Again in humans, a significant positive correlation was reported between recovery of motility after freeze-thawing and SOD content in sperm from the 90% gradient pellet containing highly purified mature sperm. However, in the same study there was also a significant negative correlation between motility after thawing and SOD content in the unfractionated sample [33]. In our study, SOD was measured in seminal plasma; therefore, an increase in SOD activity during storage could support the suggestion that increased SOD activity is a result of leakage of intracellular enzyme from sperm cells to seminal plasma.

The significant correlations between SOD in fresh seminal plasma and semen parameters after 3 days of storage led us to evaluate the ability of the former to predict semen quality on day 3. According to ROC analysis, it could be

used to predict semen quality after 3 days of liquid storage in terms of progressive motility (AUC = 0.86; $P < 0.001$) and viability (AUC = 0.85; $P < 0.001$). We can predict with 88.9% certainty that fresh semen samples with SOD activity less than 1.22 U/mL will retain more than 25% progressive motility and, with 100% certainty, that fresh semen samples with activities of SOD less than 1.26 U/mL will retain more than 85% of viable spermatozoa after storage. TNF- α could also be used to predict the viability of boar semen following storage [21]. A cut-off value of 150 pg/mL would allow the prediction, with 92.35% certainty, that fresh semen samples with more than 150 pg/mL of TNF- α in seminal plasma will retain more than 85% of viable spermatozoa following 3 days of storage [21].

Diagnostic evaluation based on fulfilling four criteria for satisfactory semen characteristics after storage provided a higher prognostic value of SOD than similar evaluation based on individual semen parameter. It is important to know what percentage of samples with positive test result will not fulfil all criteria for satisfactory semen characteristics after 3 days of storage. The optimal cut-off value of SOD enabled the prediction, with 100% certainty, that fresh semen samples with SOD more than 1.05 U/mL will not fulfil criteria stated above. On the other hand, it can be predicted, with 87.5% certainty, that fresh semen samples with less than 1.05 U/mL will exhibit the required quality characteristics after storage. In our recent study, semen parameters for the prediction of boar semen quality following short storage were evaluated according to individual parameters. It was found that progressive motility could be predicted with at least 80% accuracy from progressive motility, normal morphology, and acrosome abnormalities of fresh semen samples [21].

5. Conclusions

SOD in seminal plasma of fresh boar semen was found to be a suitable predictive marker for progressive motility and viability following 3 days of storage. Moreover, SOD was a valuable indicator of semen quality following storage when looking at a combination of four standard parameters: motility, progressive motility, morphology, and viability. Therefore, SOD in fresh boar seminal plasma could be a reliable and simple test for predicting semen quality after 3 days of storage. Further studies are needed to evaluate SOD in fresh seminal plasma in relation to the pregnancy outcome in sows.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Expression of Mesenchymal Stem Cells-Related Genes and Plasticity of Aspirated Follicular Cells Obtained from Infertile Women

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After removal of oocytes for *in vitro* fertilization, follicular aspirates which are rich in somatic follicular cells are discarded in daily medical practice. However, there is some evidence that less differentiated cells with stem cell characteristics are present among aspirated follicular cells (AFCs). The aim of this study was to culture AFCs *in vitro* and to analyze their gene expression profile. Using the RT² Profiler PCR array, we investigated the expression profile of 84 genes related to stemness, mesenchymal stem cells (MSCs), and cell differentiation in AFCs enriched by hypoosmotic protocol from follicular aspirates of infertile women involved in assisted reproduction programme in comparison with bone marrow-derived mesenchymal stem cells (BM-MSCs) and fibroblasts. Altogether the expression of 57 genes was detected in AFCs: 16 genes (*OCT4*, *CD49f*, *CD106*, *CD146*, *CD45*, *CD54*, *IL10*, *IL1B*, *TNF*, *VEGF*, *VWF*, *HDAC1*, *MITF*, *RUNX2*, *PPARG*, and *PCAF*) were upregulated and 20 genes (*FGF2*, *CASP3*, *CD105*, *CD13*, *CD340*, *CD73*, *CD90*, *KDR*, *PDGFRB*, *BDNF*, *COL1A1*, *IL6*, *MMP2*, *NES*, *NUDT6*, *BMP6*, *SMURF2*, *BMP4*, *GDF5*, and *JAG1*) were downregulated in AFCs when compared with BM-MSCs. The genes which were upregulated in AFCs were mostly related to MSCs and connected with ovarian function, and differed from those in fibroblasts. The cultured AFCs with predominating granulosa cells were successfully *in vitro* differentiated into adipogenic-, osteogenic-, and pancreatic-like cells. The upregulation of some MSC-specific genes and *in vitro* differentiation into other types of cells indicated a subpopulation of AFCs with specific stemness, which was not similar to those of BM-MSCs or fibroblasts.

1. Introduction

In infertile women, oocytes are retrieved by ultrasound-guided transvaginal follicular aspiration in the assisted reproduction programme. After removal of oocytes for *in vitro* fertilization, follicular aspirates which are rich in somatic follicular cells are discarded in daily medical practice. Each follicular aspirate consists of numerous types of somatic cells along with follicular fluid [1]. The main types of aspirated follicular cells (AFCs) are represented by granulosa cells (GCs) and theca cells (TCs). The main role of GCs is to support the oocyte by providing some nutrients that are essential for oocyte growth and development and to accumulate the metabolites secreted by the oocyte. On the other hand, TCs produce androgens which are converted

to estradiol by GCs [2]. Nevertheless, the follicular aspirate is also composed of other types of cells such as red and white blood cells thus reflecting good vascularization and some resident immune cells in ovarian follicles. Moreover, also some vaginal and ovarian surface epithelial cells can be present among AFCs since these tissues are penetrated during transvaginal follicular aspiration [3, 4].

Follicular aspirates are discarded in daily medical practice but could be an important source for potential research, diagnostics (e.g., immunoassays), and cell therapy in the future, since it has already been evidenced that subpopulations of AFCs can express some stem cell characteristics [5]. Especially, GCs represent a very interesting subpopulation of AFCs as demonstrated by several studies and recently reviewed by our group [6]. GCs originate from

ovarian surface epithelium and form the major part of the growing follicle, possess a remarkable proliferation activity, and represent a predominant type of AFCs [7]. Studies evidenced expression of the stemness-related marker *OCT4* and multiple mesenchymal lineage-related markers in GCs along with their differentiation into other types of cells [8], especially spontaneous differentiation into osteogenic-like cells [9]. Moreover, the possible contribution of less differentiated GCs in development of ovarian cancers has been suggested [10]. Along with GCs, it has also been shown that subpopulation of TCs contains putative stem cells [11].

It is of great scientific interest to isolate, proliferate, and research the less differentiated/progenitor cells among AFCs for potential medical use in the future. However, there have been no studies until now which would analyze the broader gene expression profile of AFCs and elucidate the potential relation of AFCs to mesenchymal stem cells (MSCs), the most common cells tested in the regeneration of impaired ovarian function in the animal models [12, 13].

The aim of this study was therefore to analyse the expression of eighty-four different genes related to stemness (pluripotency), MSCs, and cell differentiation in cultured AFCs from follicular aspirates of infertile women included in the assisted reproduction programme in comparison with bone marrow-derived MSCs (BM-MSCs) and human dermal fibroblasts (HDFs). We also tested the osteogenic, adipogenic, and pancreatic differentiation in cultured AFCs to evidence their plasticity. Our results showed that cultured AFCs expressed specific stemness related to MSCs but other than in BM-MSCs and somatic fibroblasts. Moreover, the cultured AFCs were able to differentiate into adipogenic-, osteogenic-, and pancreatic-like cells *in vitro*.

2. Materials and Methods

2.1. Collection of AFCs. This study was approved by the Slovenian Medical Ethical Committee (Ministry of Health, number 196/10/07). After written informed consents, follicular aspirates were collected by transvaginal ultrasound-guided aspiration from twelve infertile patients treated with controlled ovarian hyperstimulation for assisted reproduction. Patients were treated with various exogenous gonadotropins as described previously [14]. After removal of the cumulus oophorus-oocyte-complexes, the AFCs were enriched from the follicular aspirates using hypoosmotic technique as described by Lobb and Younglai [15], mainly to remove red blood cells. Briefly, the freshly collected follicular aspirates from each patient were pooled in conical bottomed 50 mL polypropylene centrifuge tubes and centrifuged at 1400 rpm for 6 min. The supernatant was aspirated and the remaining cell slurry was pipetted into a 15 mL conical bottomed polystyrene centrifuge tube. To the cell slurry 9.0 mL of sterile distilled water was added and the tube was capped and mixed. After 60 s, 1.0 mL of 10x concentrated phosphate buffer saline (PBS; pH 7.4) was added and the tube was capped and mixed. The tubes were then centrifuged at 800 rpm for 3 min; the supernatant was discarded; the cell pellet was resuspended in

0.5 mL of culture medium and transferred into a culture dish. From each patient, one AFCs culture was established.

2.2. Cell Cultures. Cells were cultured in gelatin-coated 4-well culture dish (15 mm well diameter) at concentration of 1×10^5 cells per well. For the culture medium, DMEM/F12 (Sigma-Aldrich) with 20% follicular fluid serum (FF) retrieved from the *in vitro* fertilization programme was used. FF was prepared as described previously by Stimpfel et al. [16]. The cells were cultured in a CO₂ incubator at 37°C and 6% CO₂ in air and daily monitored at the heat-staged inverted microscope (Nikon, Japan). When the cell culture was set up, the culture medium was replaced by a fresh medium on the next day to remove the remaining red blood cells. The cell splitting was performed when needed using 0.15% trypsin (Sigma-Aldrich). Alive AFCs were maintained in a cell culture based on two criteria: (i) cells were attached to the surface of culture dish and (ii) cells proliferated. The cells were cultured up to 2 months.

2.3. Gene Expression Analysis. Human Mesenchymal Stem Cell RT² Profiler PCR Array (PAHS-082, SABiosciences, Qiagen) was used to evaluate the expression of 84 specific genes related to stemness (pluripotency), MSCs, and cell differentiation—osteogenesis, adipogenesis, chondrogenesis, myogenesis, and tenogenesis (see Supplementary Table 1 available online at <http://dx.doi.org/10.1155/2014/508216>). After 5 days of culturing, three AFCs cultures from three different patients who aged 36 years (uterine abnormality), 36 years (no indication of infertility/male infertility), and 38 years (tubal factor of infertility) were pooled together and analysed along with control samples. As a positive control, a commercially available cell line of bone marrow-derived mesenchymal stem cells (BM-MSCs) was used (Chemicon, Millipore, cat. number SCC034). These cells were cultured in a mesenchymal stem cell expansion medium provided by the same producer (cat. number SCM015). As a negative control, adult human dermal fibroblasts (HDFs) were used (Cascade Biologicals, Invitrogen, cat. number C-013-5C), which were cultured in DMEM/F12 (Sigma-Aldrich) with 10% FBS (Gibco, Invitrogen).

The total RNA was isolated from 10^5 to 10^6 cells using the miRNeasy Mini kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from 500 ng of the total RNA using the RT² First Strand Kit (Qiagen), which includes the additional removal of genomic DNA from the RNA sample and a specific control of reverse transcription. The quality of isolated RNA was also evaluated using RT² RNA QC PCR Arrays (Qiagen) according to the manufacturer's instructions. This test includes various measures allowing to control the presence of reverse transcription and PCR inhibitors, contamination with genomic DNA, and contamination with DNA during the procedure.

After all control tests, the samples were analysed using the RT² Profiler PCR Array. Altogether, 84 different genes were simultaneously amplified in the sample. A melting curve analysis was performed to verify that the product consisted of a single amplicon. PCR arrays were performed

in 384-well plates on a LightCycler 480 instrument (Roche Applied Science). Briefly, the reaction mix was prepared from 2x SABiosciences RT² qPCR Master Mix and 102 μ L of sample cDNA. 10 μ L of this mixture was added into each well of the PCR Array. The data were analysed via Roche LightCycler 480 software and the C_t values were extracted for each gene. The thresholds and baselines were set according to the manufacturer's instructions (SABiosciences, Qiagen). The data were analysed using software supplied by Qiagen (<http://www.sabiosciences.com/pcr/arrayanalysis.php>). The fold change in gene expression (compared to positive control BM-MSCs) was calculated using the $\Delta\Delta C_t$ method. A more than threefold change in gene expression (compared to positive control BM-MSCs) was considered as the up- or downregulation of a specific gene expression.

2.4. Alkaline Phosphatase Activity Staining. An alkaline phosphatase detection kit (Millipore) was used for staining of alkaline phosphatase (AP) activity. Briefly, the AFCs were fixed in 4% paraformaldehyde (PFA) for 1 min, washed with PBS, and incubated for 15 min in a working solution of reagents, which consisted of Fast Red Violet, Naphthol AS-BI phosphate solution and water in a 2:1:1 ratio. The culture was observed under an inverted microscope (Hoffman illumination) to confirm AP activity. The cells or cell clusters expressing AP activity were stained from pink to violet.

2.5. Differentiation of AFCs into Osteogenic-, Adipogenic-, and Pancreatic-Like Cells. Osteogenic differentiation was induced using the well-known osteogenic differentiation medium [17]. It consisted of DMEM low glucose, L-glutamine, FBS, dexamethasone (Sigma), L-ascorbic acid 2-phosphate (Sigma), β -Glycerophosphate (Sigma), and penicillin/streptomycin. To confirm successful differentiation, the cell culture was stained using the von Kossa protocol after 12–14 days of differentiation. The cells were fixed in a 4% PFA, incubated in 2% silver nitrate in the dark for 10 minutes, washed with distilled water, and exposed to UV-light for 25 minutes. After washing, the cells were observed under an inverted microscope to detect the calcium deposits, which were stained black.

To induce adipogenic differentiation, an induction medium was used as previously described [16]. The cells were cultured in a medium consisting of hESC medium (DMEM/F12, 20% KnockOut Serum Replacement (Gibco), 1 mM L-glutamine (PAA), 1% nonessential amino acids (PAA), 0.1 mM 2-mercaptoethanol (Invitrogen), 13 mM HEPES, 8 ng/mL human basic fibroblast growth factor (bFGF, Sigma-Aldrich), and 1% penicillin/streptomycin) and 20% FF. The differentiation medium was changed every 3–4 days. After 2 weeks, the cells were fixed in a 4% PFA for 20 minutes and incubated for 10 minutes in an Oil Red O working solution. After thorough washes, the cells were observed under an inverted microscope for presence of lipid droplets, which were stained red.

To induce pancreatic differentiation, the cells were cultured according to the protocol of Chandra et al. [18] which was slightly modified. Briefly, the cells were cultured for two

days in SFM medium (serum free medium; DMEM/F12, 1% ITS, 1% BSA) supplemented with 4 nM activin A, 50 μ M 2-mercaptoethanol, and 2 ng/mL bFGF. On the third day, the medium was changed to SFM supplemented with 0.3 mM taurine and on the fifth day to SFM supplemented with 3 mM taurine, 1 mM nicotinamide, and 1% nonessential amino acids. After 10–14 days, the cells were analysed by using dithizone staining. Briefly, the stock solution of dithizone was prepared by dissolving 10 mg of dithizone in 1 mL of dimethyl sulfoxide (DMSO). Then, 10 μ L of stock solution was added to 1 mL of DMEM/F12 and filtered through a 0.4 μ m filter, and cells were incubated in this working solution for 15 min at 37°C. After incubation, the cells were washed 4 times with PBS and observed under an inverted microscope. Positively stained cells were coloured red.

3. Results

3.1. Expression of MSCs-Related Genes in AFCs and Fibroblasts in Comparison to BM-MSCs. Expression of 57 genes was detected in AFCs when compared with BM-MSCs (positive control) (Table 1). Sixteen genes were upregulated in AFCs, among which MSC-associated genes *IL10* and *CD45* were two of the most upregulated genes with fold change of almost 1100 and 900, respectively. Fold change between 30 and 40 was detected for MSC-specific or associated genes *CD49f*, *TNF*, *IL1B*, and adipogenesis- and osteogenesis-related *RUNX2*. Two highly upregulated genes were also MSC-specific or associated genes *CD106* and *VWF* with fold change of around 20 and 15, respectively. All other genes (*OCT4*, *CD146*, *CD54*, *VEGF*, *HDAC1*, *MITF*, *PPARG*, and *PCAF*) showed fold change between 3 and 10 (Figure 1(a)). Twenty genes were downregulated in AFCs when compared with BM-MSCs, among which MSC-specific or associated genes *COL1A1*, *MMP2*, and *PDGFRB* were the most downregulated genes with fold changes -266 (*COL1A1*), -225 (*MMP2*), and -119 (*PDGFRB*). Highly downregulated genes were also *FGF2*, *CD73*, *CD90*, *NUDT6*, *NES*, and *CD105*, with fold changes between -33 and -12, respectively. All other genes (*GDF5*, *CASP3*, *CD13*, *CD340*, *KDR*, *BDNF*, *IL6*, *BMP6*, *SMURF2*, *BMP4*, and *JAG1*) showed fold change between -3 and -10 (Figure 1(b)). There were 27 genes which were not detected in AFCs; about one-third of them was stemness or MSCs-specific genes; one-third was genes associated with MSCs, and one-third was osteogenesis- or chondrogenesis-related genes. All these data showed that cultured AFCs expressed several genes specific or associated with MSCs, but the expression pattern was different than in BM-MSCs. Similar to BM-MSCs, AFCs did not express the key genes related to stemness or pluripotency (*SOX2*, *REX1*, *TERT*, *WNT3A*, and *INS*) or expressed them at very low level (*OCT4* and *LIF*).

In AFCs, there was a higher number of upregulated genes than in HDFs (negative control) in comparison with BM-MSCs. In AFCs, other set of MSC-specific or associated genes (*CD49f*, *CD106*, *CD146*, *CD45*, *CD54*, *IL10*, *IL1B*, *TNF*, *VEGF*, and *VWF*) were prominently upregulated than in HDFs (*CD90* and *KITLG*). In HDFs, the expression of lower number of genes was detected than in AFCs.

TABLE 1: Expression levels of 84 genes in adult human dermal fibroblasts and aspirated follicular cells in comparison with bone marrow-derived mesenchymal stem cells, respectively.

Gene name	Expression level		REFSEQ (mRNA)
	Fibroblasts	Aspirated follicular cells	
<i>ABCB1</i>	—	—	NM_000927
<i>ANXA5</i>	2.17	−1.50	NM_001154
<i>BDNF</i>	−16.45	−8.40	NM_001143805
<i>BGLAP</i>	−1.79	−1.49	NM_199173
<i>BMP2</i>	—	—	NM_001200
<i>BMP4</i>	2.01	−8.06	NM_001202
<i>BMP6</i>	−5.82	−9.32	NM_001718
<i>BMP7</i>	—	—	NM_001719
<i>CASP3</i>	−1.44	−3.36	NM_004346
<i>CD105</i>	−2.64	−12.04	NM_000118
<i>CD106</i>	—	19.56	NM_001078
<i>CD11c</i>	—	—	NM_000887
<i>CD13</i>	1.61	−4.93	NM_001150
<i>CD133</i>	—	—	NM_001139319
<i>CD146</i>	—	5.98	NM_006500
<i>CD15</i>	—	—	NM_002033
<i>CD166</i>	−7.06	1.36	NM_001243280
<i>CD271</i>	—	—	NM_002507
<i>CD29</i>	1.77	1.17	NM_002211
<i>CD340</i>	−1.65	−5.17	NM_001005862
<i>CD349</i>	—	—	NM_003508
<i>CD44</i>	1.51	−2.55	NM_000610
<i>CD45</i>	—	891.44	NM_001267798
<i>CD49f</i>	1.01	37.53	NM_000210
<i>CD51</i>	−1.55	−1.71	NM_001144999
<i>CD54</i>	1.15	4.79	NM_000201
<i>CD73</i>	−1.03	−23.26	NM_001204813
<i>CD90</i>	19.70	−32.90	NM_006288
<i>COL1A1</i>	−9.06	−266.87	NM_000088
<i>CSF2</i>	—	—	NM_000758
<i>CSF3</i>	—	—	NM_000759
<i>CTNNB1</i>	2.35	1.84	NM_001098209
<i>EGF</i>	—	—	NM_001178130
<i>FGF10</i>	—	—	NM_004465
<i>FGF2</i>	−1.45	−21.71	NM_002006
<i>FUT1</i>	—	—	NM_000148
<i>GDF15</i>	−19.70	−1.89	NM_004864
<i>GDF5</i>	11.88	−5.28	NM_000557
<i>GDF6</i>	—	—	NM_001001557
<i>GDF7</i>	—	—	NM_182828
<i>GTF3A</i>	−2.00	1.82	NM_002097
<i>HAT1</i>	1.23	1.75	NM_001033085
<i>HDAC1</i>	3.48	3.58	NM_004964
<i>HGF</i>	—	—	NM_000601
<i>HNF1A</i>	—	—	NM_000545
<i>IFNG</i>	—	—	NM_000619

TABLE 1: Continued.

Gene name	Expression level		REFSEQ (mRNA)
	Fibroblasts	Aspirated follicular cells	
<i>IGF1</i>	—	—	NM_000618
<i>IL10</i>	—	1074.91	NM_000572
<i>IL1B</i>	−41.07	33.83	NM_000576
<i>IL6</i>	−27.67	−4.76	NM_000600
<i>INS</i>	—	—	NM_000207
<i>JAG1</i>	−1.44	−9.99	NM_000214
<i>KDR</i>	−12.04	−3.25	NM_002253
<i>KITLG</i>	3.97	−1.87	NM_000899
<i>LIF</i>	—	−1.20	NM_001257135
<i>MITF</i>	1.63	4.82	NM_000248
<i>MMP2</i>	1.97	−225.97	NM_001127891
<i>NES</i>	−48.50	−16.00	NM_006617
<i>NOTCH1</i>	—	—	NM_017617
<i>NUDT6</i>	−1.49	−11.88	NM_007083
<i>OCT4</i>	2.27	4.63	NM_001173531
<i>PCAF</i>	4.23	6.11	NM_003884
<i>PDGFRB</i>	2.30	−119.43	NM_002609
<i>PIGS</i>	−1.49	2.48	NM_033198
<i>PPARG</i>	1.80	7.67	NM_005037
<i>PTK2</i>	1.04	−1.14	NM_001199649
<i>REX1</i>	—	—	NM_174900
<i>RHOA</i>	−1.15	−1.52	NM_001664
<i>RUNX2</i>	1.68	30.70	NM_001015051
<i>SLC17A5</i>	2.25	−1.51	NM_012434
<i>SMAD4</i>	4.11	2.06	NM_005359
<i>SMURF1</i>	1.14	1.23	NM_001199847
<i>SMURF2</i>	−2.51	−8.75	NM_022739
<i>SOX2</i>	—	—	NM_003106
<i>SOX9</i>	—	1.72	NM_000346
<i>TBX2</i>	—	—	NM_005994
<i>TERT</i>	—	—	NM_001193376
<i>TGFB1</i>	−1.54	2.03	NM_000660
<i>TGFB3</i>	1.01	1.28	NM_003239
<i>TNF</i>	—	35.75	NM_000594
<i>VEGF</i>	−4.44	3.10	NM_001025366
<i>VIM</i>	−1.53	−1.87	NM_003380
<i>VWF</i>	—	14.42	NM_000552
<i>WNT3A</i>	—	—	NM_033131

—: expression of the gene was not detected.

The expression of 50 genes was detected in HDFs when compared with BM-MSCs (Table 1). A lower number −6 genes were upregulated in HDFs, among which MSC-specific *CD90* was the most upregulated gene with fold change of around 20. All other upregulated genes (*KITLG*—associated with MSCs, *HDAC1*—osteogenesis, *PCAF*—chondrogenesis, and *SMAD4*—tenogenesis) had fold change of around 4, with exception of chondrogenesis-related *GDF5*, which had fold change of around 10 (Figure 2(a)). Ten genes were downregulated in HDFs when compared with BM-MSCs,

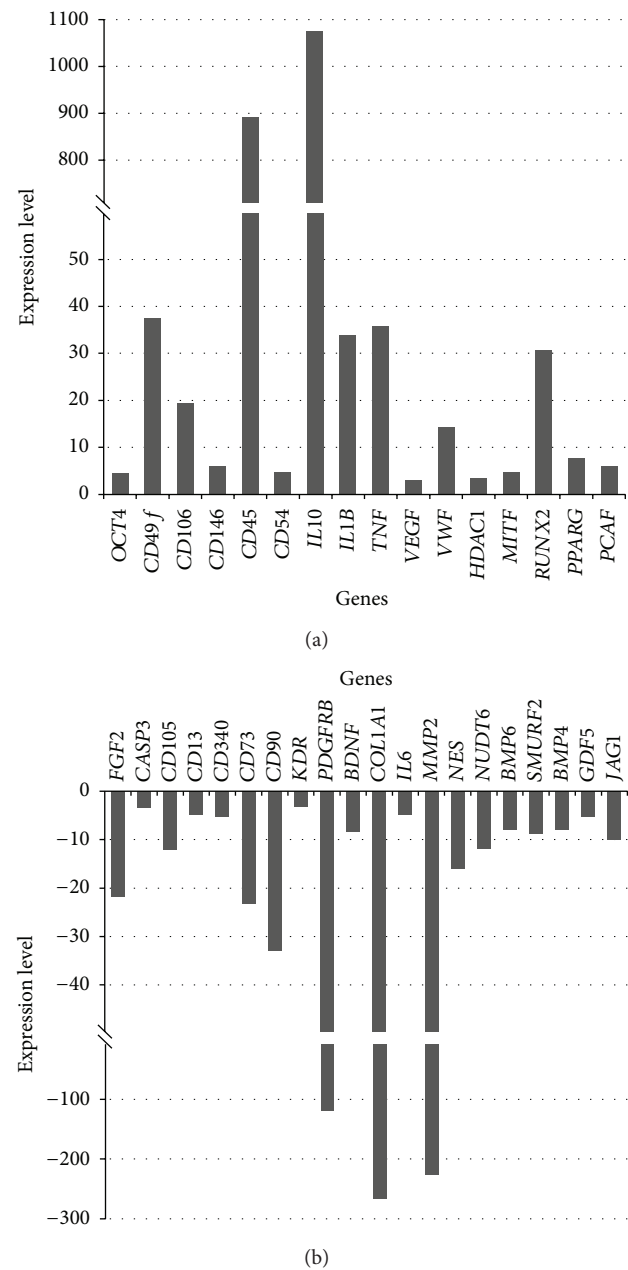


FIGURE 1: Expression levels of upregulated (a) and downregulated (b) genes in aspirated follicular cells obtained from follicular aspirates when compared with bone marrow-derived mesenchymal stem cells (positive control).

among which MSC-specific *NES*, *IL1B*, and *IL6* were the most downregulated genes with fold change of around -30 (*IL6*), of around -40 (*IL1B*), and of around -50 (*NES*). Fold change between -10 and -20 was detected for *GDF15*, *BDNF*, and *KDR* genes. All other genes (*CD166*, *COL1A1*, *VEGF*, and *BMP6*) showed fold change between -10 and -3 (Figure 2(b)).

3.2. Culturing of AFCs and Differentiation in Other Cell Types. Immediately after transferring enriched AFCs from follicular

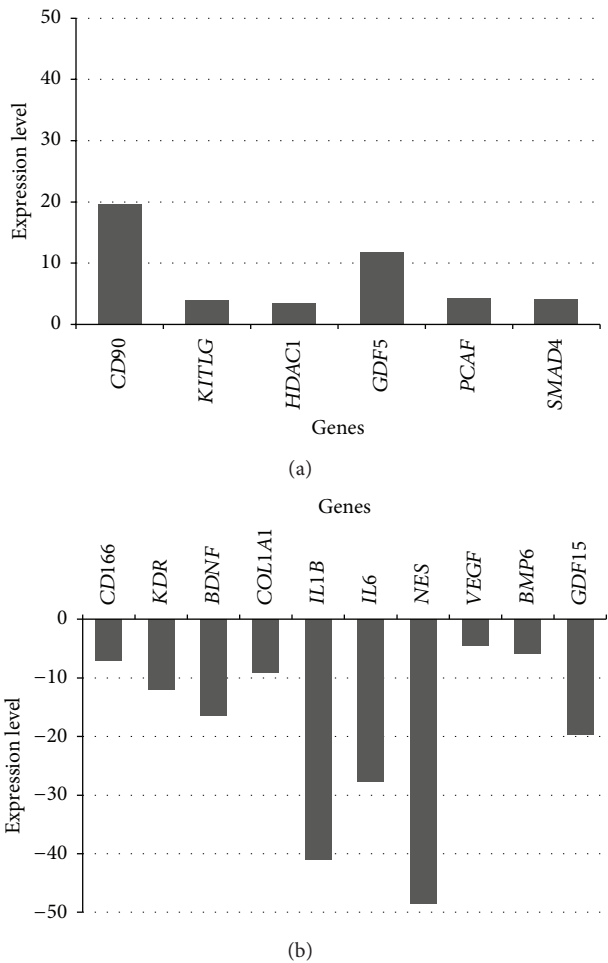


FIGURE 2: Expression levels of upregulated (a) and downregulated (b) genes in adult human dermal fibroblasts (negative control) when compared with bone marrow-derived mesenchymal stem cells (positive control).

aspirates into culture dish, we observed clusters of AFCs with approximately 100 μ m in diameter and also single AFCs with numerous surrounding red blood cells (Figure 3(a)) which were not removed with hypoosmotic protocol. After AFCs were attached to a culture dish surface, red blood cells were removed upon washing with PBS and first change of the culture medium (on the second day). AFCs exhibited fibroblast-like phenotype (Figure 3(b)), although epithelial-like AFCs were also observed in minority. After 48 hours, AFCs also started migrating from packed clusters. We were able to maintain AFCs alive for 2 months; however, viability (attachment to the surface and cell proliferation) of AFCs decreased with every passage, but it was unique case with every patient.

Cultured AFCs were highly positive for AP, and around 60% AFCs showed strongly pink-violet staining (Figure 3(c)) throughout the culturing. When AFCs were exposed to media for osteogenic differentiation, cell morphology was slightly changed; they shrunk, and around 10% of AFCs

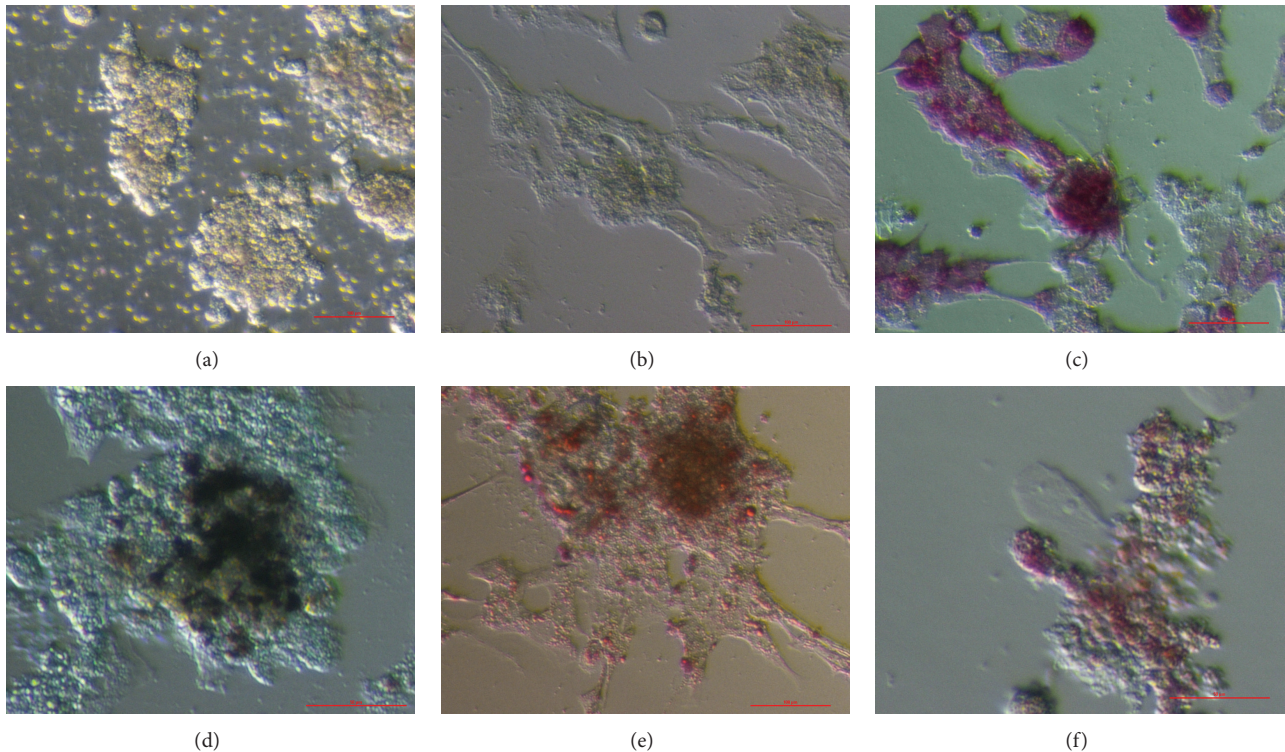


FIGURE 3: Epithelial-like phenotype of aspirated follicular cells (AFCs) in culture dish immediately after enrichment with hypoosmotic method (a). Fibroblast-like phenotype of AFCs in culture dish 48 hours after isolation (b). AFCs positive for alkaline phosphatase activity (pink-violet) (c). Differentiation of AFCs into osteogenic-like cells, von Kossa-positive staining (d). Differentiation of AFCs into adipogenic-like cells, accumulation of lipid droplets (dark red) (e). Differentiation of AFCs into pancreatic-like cells, dithizone-positive (bright red-pink) cell cluster (f). Scale bar: 100 μ m (a, b, c, e) and 50 μ m (d, f).

stained positively for mineralization (Figure 3(d)). Additionally, when AFCs were exposed to media for adipogenic differentiation, accumulation of lipid droplets was observed throughout the cell culture (Figure 3(e)). AFCs were also exposed to media for pancreatic differentiation. Cell morphology was changed forming clusters of islet-like structures and around 5% of cells positively stained on dithizone (Figure 3(f)).

4. Discussion

In this study, AFCs obtained from follicular aspirates of infertile women included into the *in vitro* fertilization programme were successfully cultured and their stemness was confirmed. The gene expression profile and *in vitro* differentiation of cultured AFCs into other cell types confirmed the relation of AFCs to MSCs, but their stemness was specific and it differed from BM-MSCs and fibroblasts.

The *in vitro* culturing and research of molecular and cellular characteristics of AFCs and their subpopulations such as GCs or TCs are still difficult since there is no ultimate protocol for their purification from follicular aspirates. Subpopulations of AFCs can be isolated by flow cytometry based on the expression of specific cell marker, for example, follicle-stimulating hormone receptor (FSHR) for isolation of GCs [8]; however, this approach can lead to a loss of less

differentiated/progenitor GCs which do not express FSHR yet. In this study, we used the hypoosmotic purification protocol described by Lobb and Younglai [15] to enrich AFCs because it is quite simple and can be quickly done, removes most of red blood cells from the sample, and yields more AFCs in comparison with multistep protocols. The follicular aspirates also contain a proportion of white blood cells which represent approximately 15% of all cells [19] and are unavoidable contaminant. On the other hand, these “contaminating” cells could play an important role in maintaining a more physiological ovarian stem cell niche [20].

In this study, we successfully established a long-term culture of AFCs. In previous studies, the apoptosis represented a major problem in AFCs culturing and research. However, we found for the first time that the addition of follicular fluid serum to the culture medium enables a long-term survival of AFCs *in vitro*. Because the potential use of AFCs is related to their culture and proliferation *in vitro*, we were interested in gene expression analysis of cultured AFCs more than freshly isolated. However, *in vitro* culturing can significantly affect the gene expression of cells as previously shown in human stromal cells [21]. Even more, for some AFCs like GCs, it has been demonstrated that they can undergo dedifferentiation *in vitro* and downregulation of GCs-specific genes may occur after 96 hours of culturing [9].

Our data showed that all three groups of analyzed cells expressed a proportion of MSC-specific or associated genes thus reflected the same—mesodermal—origin of cells. In spite of that, the gene expression profile of AFCs, BM-MSCs, and HDFs was different and indicated three distinct groups of cells. There were eight genes which were expressed in both the AFCs and BM-MSCs, but were not expressed in HDFs; these genes were related to stemness (*LIF*) and were MSCs-specific (*CD106* and *CD146*), associated with MSCs (*IL10*, *CD45*, *TNF*, and *VWF*) or chondrogenesis related (*SOX9*). In AFCs, several MSCs-specific or associated genes were upregulated. The AFCs were not only characterized by a very high expression of genes *IL10* and *CD45* that may reflect their association with MSCs, but also to a lower extent the contamination with blood cells. The gene *IL10* is known to be related to immunoregulation (inflammation), while the gene *CD45* encodes the protein belonging to the tyrosine phosphatase (PTP) family; the PTPs are known to be signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitosis, and oncogenic transformation according to the GeneCard database.

The results of this study show that AFCs expressed several genes typical for somatic ovarian cells, especially GCs. In addition, the morphology of AFCs clusters resembled the GCs; therefore, it is not excluded that GCs represented majority of cells in our cell cultures. The expression of gene *VEGF*, vascular endothelial growth factor, was previously demonstrated in GCs and was shown to be very important factor in controlling angiogenesis during development of *corpus luteum* [22]. In addition, *CD146*, melanoma cell adhesion molecule, was shown to be expressed on human luteinizing GCs [23]. *CD49f*, also known as integrin alpha-6, has been demonstrated to be expressed on the surface of human GCs and represents a differentiation marker of GCs [24]; it was found to be more distinctive for GCs from the inner layers of follicle [25]. The gene *PPARG*, peroxisome proliferator-activated receptor gamma, encodes a nuclear hormone receptor which is related to steroid hormone action [26]. The activity of GCs is strongly influenced by follicle-stimulating hormone and luteinizing hormone [27]. The gene *HDAC1*, histone deacetylase 1, is one of the important regulators of human luteinizing hormone receptor gene transcription [28]. In AFCs, also some genes related to osteogenesis and adipogenesis were upregulated; *MITF* has been connected with osteogenesis [29], along with *RUNX2* [30]. In addition, *PCAF* was recently shown to acetylate *RUNX2* which leads to transcriptional activity and thus promotes osteoblast differentiation [31]. In AFCs, there was a higher number of upregulated genes related to MSCs than in HDFs in comparison with BM-MSCs and other set of MSC-specific or associated genes was prominently upregulated than in HDFs. In addition, the genes upregulated in HDFs were more related to cell differentiation (osteogenesis, chondrogenesis, tenogenesis) than to stemness thus indicating that HDFs were more differentiated cells than cultured AFCs.

The AFCs were not pluripotent stem cells, because they did not express genes related to pluripotency such as *REX1*, *SOX2*, *TERT*, and *WNT3A*. In spite of that, they expressed two pluripotency-related genes: *OCT4* and *LIF* to a lower

extent. The expression of *OCT4* in AFCs probably reflects the presence of GCs as previously confirmed by other studies [8, 32, 33]. However, *OCT4* was also expressed in both BM-MSCs and HDFs to the same extent; therefore, the nonspecificity of primer for *OCT4A*, related to pluripotent stem cells [34], is not excluded. It needs to be exposed that the *LIF* gene, an important marker of stemness [35], was detected to the same extent in AFCs and BM-MSCs, but was not detected in HDF; this might reflect a lower stemness of HDFs.

A subpopulation of AFCs expressed a degree of plasticity, because we were able to successfully differentiate them into osteogenic, adipocyte and pancreatic-like cells. AFCs seem to be especially in favour of osteogenesis thus reflecting the presence of GCs, as evidenced by other studies [9, 36]. In our experiments, AFCs strongly expressed the gene *RUNX2* which is involved in osteogenesis [37] and GCs luteinization [38], differentiated into osteogenic-like cells confirmed by Von Kossa staining and stained positively for alkaline phosphatase activity which is considered as an early marker of osteogenesis [39]. Moreover, AFCs were successfully differentiated into adipose and pancreatic-like cells in this study. To our knowledge differentiation of AFCs into adipocyte and pancreatic-like cells has not been reported until now; therefore, our work additionally supports the idea about the stemness and plasticity of human AFCs.

5. Conclusion

In conclusion, the results of our study showed that AFCs enriched from follicular aspirates of infertile women using hypoosmotic protocol and cultured *in vitro* expressed 57 from 84 analyzed genes related to stemness, MSCs, and cell differentiation. Numerous upregulated genes were specific for MSCs or were associated with them. The expression of these genes confirmed the stemness of AFCs in our cultures; however, the gene expression profile differed from that of BM-MSCs. The gene expression profile of AFCs also differed from that of HDFs which were found to be more differentiated cells. In AFCs, also several expressed genes were related to the ovary and its function. The AFCs expressed a degree of plasticity and were successfully differentiated into other types of cells which are otherwise not present in the ovary.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Fetus Sound Stimulation: Cilia Memristor Effect of Signal Transduction

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Background. This experimental study evaluates fetal middle cerebral artery (MCA) circulation after the defined prenatal acoustical stimulation (PAS) and the role of cilia in hearing and memory and could explain signal transduction and memory according to cilia optical-acoustical properties. **Methods.** PAS was performed twice on 119 no-risk term pregnancies. We analyzed fetal MCA circulation before, after first and second PAS. **Results.** Analysis of the Pulsatility index basic (PIB) and before PAS and Pulsatility index reactive after the first PAS (PIR 1) shows high statistical difference, representing high influence on the brain circulation. Analysis of PIB and Pulsatility index reactive after the second PAS (PIR 2) shows no statistical difference. Cilia as nanoscale structure possess magnetic flux linkage that depends on the amount of charge that has passed between two-terminal variable resistors of cilia. Microtubule resistance, as a function of the current through and voltage across the structure, leads to appearance of cilia memory with the “memristor” property. **Conclusion.** Acoustical and optical cilia properties play crucial role in hearing and memory processes. We suggest that fetuses are getting used to sound, developing a kind of memory patterns, considering acoustical and electromagnetically waves and involving cilia and microtubules and try to explain signal transduction.

1. Introduction

Prenatal acoustical stimulation (PAS) is a method of early detection of development of fetal auditory and behavioral function. It is based on a method of detecting fetal reactions on the defined sound stimulation [1]. However, the first idea of this method was proposed twenty years ago [2].

This experimental study evaluates fetal middle cerebral artery (MCA) circulation after the defined prenatal acoustical stimulation (PAS), the role of cilia in hearing and memory and explains signal transduction and memory according to optical and acoustical properties of cilia. Acoustical and

optical (electromagnetic) properties of cilia play crucial role in this process. Under the influence of clustering water and interaction with microtubules, not only may cilia signal transduce from acoustical to electrical but also may have memory property.

Cilia are microtubule-based organelle, tiny hair-like structure that performs feats such as clearing microscopic debris from the lungs and determining the correct location of organs during development. Due to the importance of cilia functions for health, there is great interest in understanding the mechanism that controls the cilia beating patterns and signal transduction. Transduction of acoustical to electrical

signal on molecular and organelles levels is challenging and is not well understood yet. Auditory and vestibular inner ear hair cells convert the mechanical stimuli (acoustical, gravity, and head movement) into electrical signals [3]. This mechanotransduction process is initiated by opening of mechanosensitive cationic channels near the tips of hair cell stereo cilia, but the identity of these ion channels is still unknown [4]. We assume that cilia, as one of the first natural biological devices with efficient signal transduction, play crucial role in signal transduction.

Mutations in over 30 different genes can lead to cilia defects and complex interactions among ciliopathy-associated proteins [5]. Genetic mutations compromising the proper cilia functioning are associated with defects in ciliogenesis and they form new class of diseases called ciliopathies [6].

They can affect many different organs and cause polycystic kidney disease [7], primary cilia dyskinesia, retinitis pigmentosa, polydactyly, brain malformations and hydrocephalus [8–11], blindness, anosmia [12], asthenospermia [13], obesity [14], cognitive deficits [15], and some syndromes: Bardet-Biedl syndrome [15–17], Kartagener's syndrome [16, 18, 19], and Senior-Løken syndrome [16]. There are also indications that the primary cilium is important in oncogenesis [15, 20], behavioral [21] and mental disorders [22]. Some findings imply that primary cilia play important roles in the earliest stages of embryonic development, which could be important in regenerative medicine [20].

The cilium is an organelle with chemosensory, photosensory, and mechanosensory function in various body tissues and it plays an important role in normal development. We wanted to give the new insight to its memory function, based on our current experiment and our previous mathematical model of converting acoustical to electrical signal [23] which results in changes of the cell behavior and physiology, according to primitive memory effect.

2. Materials and Methods

Our study included 119 healthy pregnant women with no risk pregnancies, without complications of any kind, with the delivery at time. Our examination was performed in period from 37 to 41 weeks of gestational age. Gestational age was determined in relation to last menstruation and estimated by ultrasound examination. We examined 271 pregnant patients, but 52 of them did not meet inclusion criteria because the gestational week was beyond 37 gestational weeks. All women were informed about our procedures and they gave written informed consent before enrollment. Our examination has research Ethics Board Approval of Department of Obstetrics and Gynecology "Narodni front," Belgrade, Serbia. Experimental study has been organized as a part of prospective experimental trial under the supervision of Ministry of Health and Education of Republic of Serbia, (2011–2014). Project included Belgrade University Medical School, Belgrade, Serbia (Department of Obstetrics and Gynecology "Narodni front" and Institute for Gynecology and Obstetrics, Clinical Center of Serbia), and Institute for Experimental Phonetics and Speech Pathology, Belgrade, Serbia. Modeling of microtubule and cilia biophysical properties has been done

at the Department of Biomedical Engineering, Faculty of Mechanical Engineering, University of Belgrade, Serbia.

Fetal examination starts with standard ultrasound examination. Noise-canceling headphones types EP-107 are put on women's head, to cancel the influence of mother's acoustical stimulation. Fetal head and ear position near the mother's abdominal wall are determinate and the speaker is positioned 5 cm from abdominal wall, to the direction of fetal ear. The circle of Willis is easy to identify with B-scan and blood flow using color Doppler. Using data of blood flow through fetal middle cerebral artery (MCA), Pulsatility index before sound stimulation (PIB) can be measured [24].

The fetus is exposed to the digitalized generated sound stimulus performed by loudspeaker sets 5 cm away from abdominal wall. This sound is 90 dB of intensity, frequency range is 1500–4500 Hz, and the duration is 0,2 s. This sound stimulus is presenting once in order to investigate changes in cerebral circulation of the fetus. Color Doppler ultrasound is used to identify middle cerebral artery flow after sound stimulation and identify the PI index after it. We also measure the time in seconds from the stimulus to the measured values of Pulsatility index after stimulation (PIR) and identify reactivity. Doppler analysis of blood flow is performed on the Toshiba Nemio with the possibility of Doppler and color Doppler and convex sector probe with the frequency of 3.5 MHz. We examined blood flow, which is registered in the first third of middle cerebral artery from branching. For the analysis of the wave, we used Pulsatility index (PI):

$$PI = \frac{(S - D)}{M}, \quad (1)$$

one of the major Doppler-parameter [25]. Pulsatility index (PI) is equal to systolic (S) minus diastolic (D) amplitude value of arterial waveform, divided by the mean (M) value of the area under the waveform. This parameter is considered as an indicator of the size of the peripheral resistance and belongs to one of the Doppler-indexes of peripheral vascular resistance. Measured values of PI before (PIB) and few seconds after exposure to define digitalized generated sound stimulation (PIR 1) indicate changes in the fetal cerebral circulation. If the PI values after acoustical stimulation (PIR) are lower compared to the basic values of this index before stimulation (PIB), there is an increase in blood flow of fetal middle cerebral, while in the case of higher values of PIR compared to basic ones (PIB) signify reduction in blood flow in examined middle cerebral artery [1].

Five minutes after first stimulation, we exposed fetus to the same sound stimulus and measured the Pulsatility index (PIR 2) of the fetal MCA.

We tested the changes of PI values, basic one (PIB), after first (PIR 1) and after second stimulation (PIR 2), because we assume that fetuses develop own memory functions and did not react on the repeated stimulus in the same way. We believe that cilia and microtubules as acoustical and electrical organelles are responsible for signal transduction with memory and learning properties [26].

Statistical analysis of two-way comparisons was done using 2-tailed Student's t -tests. P values of less than 0.05 were considered significant.

3. Results

Study included 119 healthy pregnant women with no risk pregnancies, without complications of any kind, with the delivery at time. Our examination was performed in period from 37 to 41 weeks of gestational age.

The mean gestational age in weeks is 39.02. Age of pregnant women showed that the mean age is 30.2 years and the median is 30. The eldest one was 44 years old and the youngest was 18 years old. Only 0.8% of patients were younger than 20 years and 1.7% elder than 40. The majority of patients (69.7%) is at the age between 25 and 34 and could represent our population.

The analysis of the Pulsatility index basic (PIB) of fetal middle cerebral artery (MCA), before PAS, has shown that the mean PIB value is 1.4747 and median is 1.47. Maximal PIB value is 2.59, and minimal one is 0.78.

Pulsatility index reactive after the first sound stimulation (PIR 1) of fetal MCA has shown that the mean PIR 1 value is 1.3644 and median is 1.33. Maximal PIR 1 value is 2.57, and minimal one is 0.76.

Pulsatility index reactive after the second sound stimulation after five minutes (PIR 2) of fetal MCA has shown that the mean PIR 2 value is 1.4605 and median is 1.46. Maximal PIR 2 value is 2.47, and minimal one is 0.8.

Comparison of the Pulsatility index basic (PIB) and Pulsatility index reactive after the first sound stimulation (PIR 1), using *t*-test ($t = 4.445$, $P < 0.01$), showed that there is high statistical difference. It means that there is a high influence of first sound stimulation on the fetal brain circulation. Values of PIB and PIR 1 of fetal MCA are graphically presented at Figure 2.

Statistical analysis of the Pulsatility index basic (PIB) before the sound stimulation and Pulsatility index reactive after the second sound stimulation (PIR 2) of fetal MCA, according to *t*-test ($t = 1.506$, $P > 0.05$), has shown that there is no statistical difference (Figure 3).

We suggest that our fetuses are getting used to the sound stimulus, developing a kind of memory patterns, which are connected acoustical and electromagnetic waves involving cilia and microtubules.

Statistical analysis of the Pulsatility index reactive after the first sound stimulation (PIR 1) and Pulsatility index reactive after the second sound stimulation (PIR 2) of fetal MCA, using *t*-test ($t = -5.347$, $P < 0.01$), has shown that there is high statistical difference. It means that reaction after second sound stimulation is different, because fetuses from no risk pregnancies get used to it and could develop memory patterns.

4. Discussion

Cilia are microtubule-based organelles surrounded by membranes that protrude from the cells [6]. Inside cilia is a microtubule-based cytoskeleton which acts as scaffolding for various protein complexes and provides binding sites for molecular motor proteins such as kinesin II that help carry proteins up and down the microtubules [27].

Cilia are classified into three categories: motile, primary, and nodal [16].

- (1) Motile cilia are situated on the surface of nearly every cell of mammalian body and they are involved in fluid and cell movement (mucociliary clearance in the lung, cerebrospinal fluid movement in the brain, and ovum and sperm transport along the reproductive tracts). Motile cilia consist of 9 doublet microtubules surrounding 2 inner singlet microtubules (9+2), used to generate forces to induce motility [28].
- (2) Primary cilia are solitary organelles projecting from the surface of cells and they lack the central pair of microtubules needed to generate motile force, so they are described as having a 9+0 pattern. Primary cilia on epithelial cells provide chemosensation, thermosensation, and mechanosensation of the extracellular environment by playing "a sensory role mediating specific signaling cues, including soluble factors in the external cell environment, a secretory role in which a soluble protein is released to have an effect downstream of the fluid flow, and mediation of fluid flow if the cilia are motile" [29].
- (3) Nodal cilia are located at the node in gastrulation-stage embryos. They are solitary organelles that contain 9 + 0 microtubule architecture and possess the ability to move in a propeller-like way. Mutations that disrupt nodal cilia in mice indicate that they play essential roles in establishing signaling events required for specification of the left-right body axis in mammals [30].

Bearing in mind the complexity of cilia, most previous studies of cilia were attempting to study the signal transduction. Nine pairs of microtubules are arranged along the periphery, and one pair of microtubules is situated at the center. Microtubules are biopolymer filaments, hollow rods approximately 25 nm in outer diameter and 14 nm inner diameters. The tubular structure is made up of two polypeptides: alpha-tubulin and beta-tubulin [31]. Tubulin subunits (α , β) oscillate in microtubule protofilaments generating optical (electrical and magnetic) and acoustical oscillations [32]. Since mass of α and β subunits have small difference, only four amino acids, optical and acoustical oscillatory modes are coupling [23]. The nine outer pairs of microtubules are connected by nexin links. Also, each of the outer pairs is linked to the central pair by a radial spoke. Closer inspection of the outer microtubules shows that they have side arms of proteins, called dyneins. These proteins are actually mechanical motors and they are instrumental in cilia movement. The structure of cilia is much like a tube, and its long fibers are composed of microtubules. These microtubules often pair up to form doublets, which mathematical sign eight, since the two microtubules stick together along a line. Nine doublets form the larger ring that is known as the 9 + 2 pattern. When kinesin binds to one side of the doublets and not the other, the cilium flexes and curves, similar to the way our skeletal muscles contract. "In effect, cilium is a nanomachine composed of perhaps over 600 proteins in molecular complexes, many

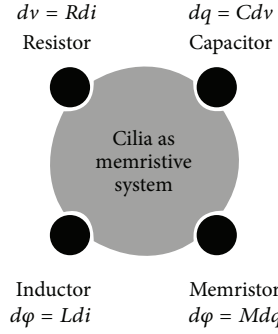


FIGURE 1: RCLM (resistance, capacitance, inductance, memory) circuit system plays crucial role in cilia dynamics (microtubules, water interaction). dv : Rdi : resistance, dq : Cdv : capacitance, $d\phi$: Ldi : inductance, and $d\phi$: Mdq : memory.

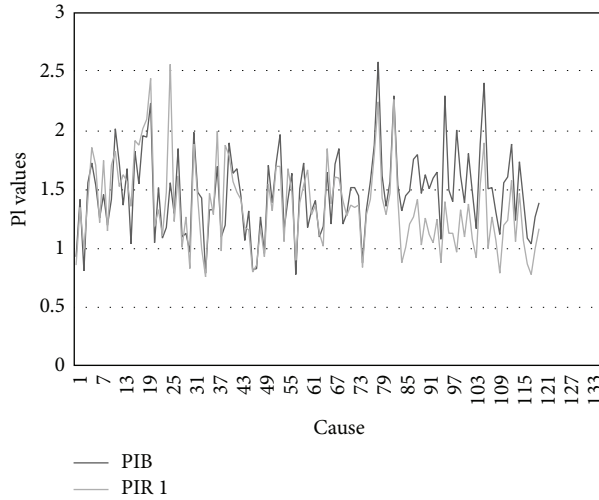


FIGURE 2: PIB and PIR 1 of fetal MCA values. PIB: Pulsatile index basic; PIR 1: Pulsatile index reactive after first sound stimulation.

of which also function independently as nanomachines” [33].

Hair cells are the sensory receptors of the organ of Corti that is located in the scala media. Two types of hair cells are present in the human cochlea: inner hair cell and the outer hair cells. Only outer hair cells are in direct contact with the tectorial membrane [34]. Ciliated columnar epithelial cells have 200 to 300 hair-like protrusions called cilia. Cells are interconnected via desmosomes and tight junctions, creating a semipermeable membrane that is more selective than membrane found in other types of cell [34]. Auditory and vestibular inner ear hair cells convert the mechanical stimuli (acoustical, gravity, and head movement) into electrical signals. This mechanotransduction process is initiated by opening of mechanosensitive cationic channels near the tips of hair cell stereo cilia, but the identity of these ion channels is still unknown [4].

New experimental system should be comprised of three main components: microtubule filaments, motor proteins called kinesin, which consume chemical fuel to move along

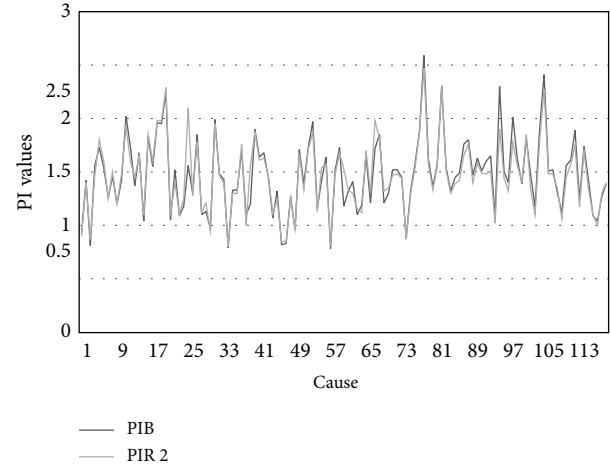


FIGURE 3: PIB and PIR 2 of fetal MCA values. PIB: Pulsatile index basic; PIR 2: Pulsatile index reactive after second sound stimulation.

microtubules, and a bundling agent that induces assembly of filaments into bundles. Based on knowledge about acoustical-electrical compatibility of cilia it is possible start to create artificial cilia-like structures that dramatically offers a new approach for cilia study and new sensory system for sensing reactions of fetus on the defined sound stimulation. The main approach to build new sensory system is to include memory effect in sensing system because cilia in process of open-close channel have “memristor” effect. By using only these three elements, resistor— R , capacitor— C , and inductor— L , this phenomenon could not be explained. The fourth element memristor M has to be included (Figure 1).

For cilia open-close channel process memristors, applied current, or voltage will cause a great change in resistance. Cilium may be characterized as switches (on-off) by investigating the time and energy that must be spent in order to achieve a desired change in resistance. Here we will assume that the applied voltage remains constant and solve for the energy dissipation during a single switching event. For a memristor to switch from R_{on} to R_{off} in time T_{on} to T_{off} , the charge must change by $\Delta Q = Q_{on} - Q_{off}$:

$$E_{switch} = V^2 \int_{T_{off}}^{T_{on}} \frac{dt}{M(q(t))} \quad (2)$$

$$= V^2 \int_{Q_{off}}^{Q_{on}} \frac{dq}{I(q)M(q)} = V^2 \int_{Q_{off}}^{Q_{on}} \frac{dq}{V(q)} = V \Delta Q.$$

To arrive at the final expression, substitute $V = I(q)M(q)$ and then $\int dq/V = \Delta Q/V$ for constant V . This power characteristic differs fundamentally from that of a microtubule-water charges which are a capacitor-based device. Unlike the transistor, the final state of the memristor in terms of charge does not depend on bias voltage.

The idea of “memristor” existence, as a fourth-elementary circuit element (resistor, capacitor, and inductor) came from Leon Chua in 1971, strongly supported in 2004 [35], and finally experimentally proved in 2008 [36]. Using water, it is shown that memristance arises naturally in clustering water

(nanoscale systems) in which cluster “solid state electronic” and ionic transport in microtubules are coupled under cilia oscillatory process. According to optical/acoustical values of cilia dynamics clustered water interacts with electrical charges on microtubules surface making RCLM (resistance, capacitance, inductance, memory) circuit system.

Fetus reactions on the defined sound stimulation in PAS can be better understood if we include cilia and microtubules as acoustical-electrical organelles. Under influence of clustering water and interaction with microtubules, not only cilia may signal transduce from acoustical to electrical, but also may have memory property. This process is self-induced by cilia. Paramecium and other one-cell organisms, which are very rich with cilia, possess both adaptive behavior and first step of learning. To explain signal transduction and memory based on cilia properties, optical/acoustical dynamics of microtubules during cilia motions is presented. Vibration spectra of microtubule indicate that cilia, as nanoscale structure (microtubules in interaction with water) possess magnetic flux linkage (Φ_m), which depends on the amount of charge q that has passed between two-terminal variable resistors of cilia (nine pairs of microtubules). In this case microtubule resistance is a function of the history of the current through and voltage across the structure. This leads to appearance of cilia memory (M) based on microtubule resistance with the “memristor,” $M = d\Phi_m/dQ$ property. Taking this knowledge about cilia properties, we are proposing new possible explanation of acoustical to electrical signal transduction under the defined sound influence on fetus during PAS. Statistical similarity of PIB and PIR 2 values, which represents no statistical changes ($t = 1.506$, $P > 0.05$) in fetal cerebral circulation after second defined acoustical stimulation is presented. This indicates that fetus auditory system possesses memory property and auditory screening is memorized.

5. Conclusions

Acoustical and optical (electromagnetic) properties of cilia play crucial rule in hearing and memory process.

Better understanding of information processing on biomolecular (microtubules), organelle (cilia), cell (hair cells) levels, and cell-nerve relationship within the organ of Corti could give us more information about fetal hearing, behavior, adaptation and memory (differences between PIB and PIR 1 and similarity between PIB and PIR 2), learning, and left-right side development.

We propose a new possible explanation of acoustical to electrical signal transduction under the defined sound influence on fetus during PAS, according to memory property of fetus auditory system.

The PAS method could be important for early detection of fetal auditory function during its development and help to establish reliable hearing screening test.

Conflict of Interest

The authors have declared that no conflict of interests exists.

Authors' Contribution

Svetlana Jankovic-Raznatovic and Svetlana Dragojevic-Dikic contributed equally in the preparation of this paper.

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Research Article

Whole Ovine Ovaries as a Model for Human: Perfusion with Cryoprotectants *In Vivo* and *In Vitro*

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These experiments were performed to test the perfusion of ovine as a model for human ovaries by cryoprotectants *in vivo* at high temperature when the permeability of capillaries is high and when blood is insensibly replaced by the solution of cryoprotectants. By our hypothetical supposition, ovaries could be saturated by cryoprotectants before their surgical removal. The objective was to examine the effectiveness of perfusion of ovine ovaries with vascular pedicle *in vivo* and *in vitro*. *Arteria ovarica* was cannuled and ovaries were perfused by Leibovitz L-15 medium + 100 IU/mL heparin + 5% bovine calf serum + 6% dimethyl sulfoxide + 6% ethylene glycol + 0.15 M sucrose + Indian ink *in vivo* and *in vitro*. In the first and second cycle of experiments, ovaries ($n = 13$ and $n = 23$) were perfused *in vivo* and *in vitro*, respectively, during 60 min with the rate of perfusion 50 mL/h (0.8 mL/min). It was established with *in vivo* perfusion that only about 10% of ovarian tissues were perfused due to an appearance of multiple anastomoses when the perfusion medium goes from *arteria ovarica* to *arteria uterina* without inflow into the ovaries. It was concluded that *in vitro* perfusion of ovine intact ovaries with vascular pedicle by freezing medium is more effective than this manipulation performed *in vivo*.

1. Introduction

Cryopreservation of whole ovine ovaries with vascular pedicles is an important focal point of research in medicine when ovine ovary is an animal model for human ovary.

In medicine, due to the increasing effectiveness of cancer treatments and good long-term prognosis for young women, the problem of postcancer infertility plays an important role. It is because chemotherapy, depending on the treatment regime chosen, can be gonadotoxic and lead to the functional death of the ovaries. The cryopreservation of ovarian tissue before cancer therapy with retransplantation after convalescence is the key to solving this problem [1–3].

In a recent publication of a research group from France, which is one of the leaders of cryopreservation of whole

mammalian ovaries [4], it is noted that cryoprotectant diffusion into perfused ovine ovaries is a potentially limiting factor that has not been adequately investigated [4].

Cryopreservation and transplantation of whole ovaries with vascular pedicle would be helpful to prevent post-transplantation ischemia. However, the data have been reported as evidence that after cryopreservation of whole ovine ovaries their viability is low [5, 6].

Development of technology of long-time perfusion of intact ovaries by cryoprotectants at low temperatures is important because earlier it was established that 24 h cooling to 5°C before cryopreservation is beneficial for freezing human ovarian fragments [7]. It was noted that the quality of follicles and the intensiveness of neovascularisation observed

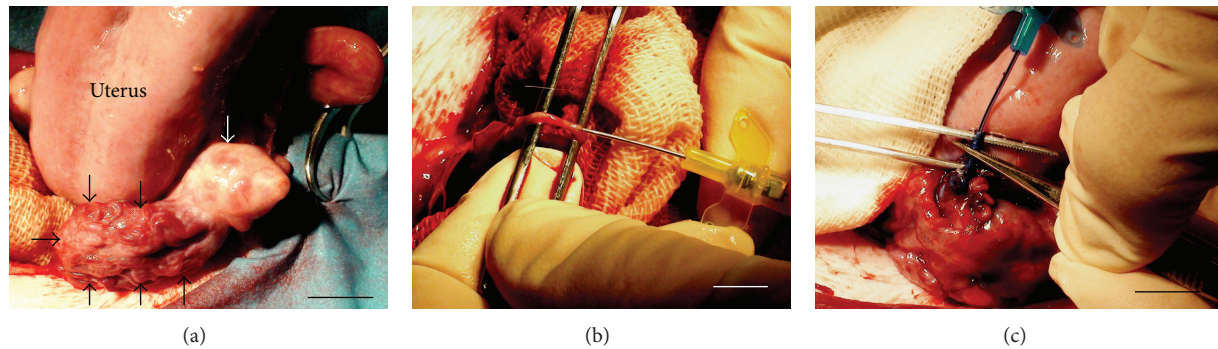


FIGURE 1: *In vivo* perfusion of ovine intact ovary with freezing medium. (a) Expelling of ovary (white arrow) with pedicle (black arrow) to operation field, (b) perforation of *arteria ovarica* by 24 G catheter for perfusion with freezing medium, and (c) beginning of perfusion of ovary with freezing medium through *arteria ovarica* with 18 G catheter. Bar = 1 cm.

in ovarian tissue precooled before culture and precooled before cryopreservation were drastically increased [7].

These experiments were performed to test the perfusion of human ovaries by cryoprotectants *in vivo* at high temperature when the permeability of capillaries is high and when blood will be insensibly replaced by the solution of cryoprotectants. Our speculation for performing experiments was the following: *in vivo* perfusion of ovaries by cryoprotectants before their removal and freezing can be more successful in comparison with this procedure *in vitro*. By our hypothetical supposition, ovaries could be saturated by cryoprotectants before their surgical removal.

The aim of this research was to study the effectiveness of perfusion of whole ovine ovaries with vascular pedicle *in vivo* and *in vitro*.

2. Materials and Methods

Except where otherwise stated, all chemicals were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

Our experiments were performed under a protocol approved by the University Ethics Board. It was used for the ovaries from Sarda animals with the body weight from 44 to 51 kg.

Freezing medium included Leibovitz L-15 medium + 100 IU/mL heparin + 5% bovine calf serum + 6% dimethyl sulfoxide (DMSO) + 6% ethylene glycol + 0.15 M sucrose + 20% Indian ink. Experimental perfusions were performed during 60 min with the rate of perfusion 50 mL/h (0.8 mL/min).

2.1. *In Vivo* Perfusion. Ovary ($n = 13$) with pedicle was expelled to operation field (Figure 1(a)). Then *arteria ovarica* was perforated by the needle of 24 G catheter and then 18 G catheter (Introcath Safety, B. Braun Melsungen AG, Melsungen, Germany) was fixed for perfusion with freezing medium (Figure 1(b)). *In vivo* perfusion was performed at 35°C (because ovary and pedicle are partially located inside of the body) (Figure 1(c)). The temperature of freezing medium was 37°C. Fallopian tubes were not separated, mesovarium was not removed, and uteroovarian anastomosis was not ligated.

2.2. *In Vitro* Perfusion. The conditions of our experiments allowed obtaining ovaries ($n = 23$) with pedicles for *in vitro* perfusion during 3–5 min after the slaughtering of animals. The 24 G catheter (B. Braun) through *aorta* was introduced to *arteria ovarica* and fixed inside (Figures 2(a) and 2(b)). *In vitro* perfusion was performed at room temperature (22°C).

To the end of the experiments, after 60 min of perfusion, the success rate of perfusion of ovaries was detected with analysis of images by the percentage of Indian-ink-perfused tissues (Figure 3). Perfusion was denoted as successful if about 100% of ovarian tissues were perfused by Indian ink.

Effectiveness of perfusion was evaluated by ANOVA. The level of statistical significance was set at a $P < 0.05$.

3. Results and Discussion

It was established that *in vivo* perfusion can be evaluated as unsuccessful; only about 10% of ovarian tissue was perfused (Figure 3) due to an appearance of anastomoses between *arteria ovarica* and *arteria uterina* when the perfusion medium goes from *arteria ovarica* to *arteria uterina* without inflow into the ovaries. It was technically difficult and practically not possible to close mechanically these multiple anastomoses during the perfusion.

After successful perfusion, approximately 100% of ovarian tissue and its vascular pedicle obtained blue colour (Figures 2 and 3).

3.1. Aim of Perfusion and Importance of Postthawing Storage of Capillaries. We believe that the aim of ovarian perfusion before cryopreservation is not saturation of cells by permeable cryoprotectants but the expelling of blood cells from blood vessels. In our opinion, if ovarian blood vessels and especially capillaries will be filled with coagulated blood cells before freezing, restoration of normal blood circulation in ovarian tissue after thawing will be not possible.

These blood cells cannot be frozen in vessels, together with ovarian tissue, without prefreezing separation. Technologically it is difficult to do it. Ovarian tissue and blood cells must be frozen with different methodologies, so that we have only one possibility to avoid ischemia due to the presence of

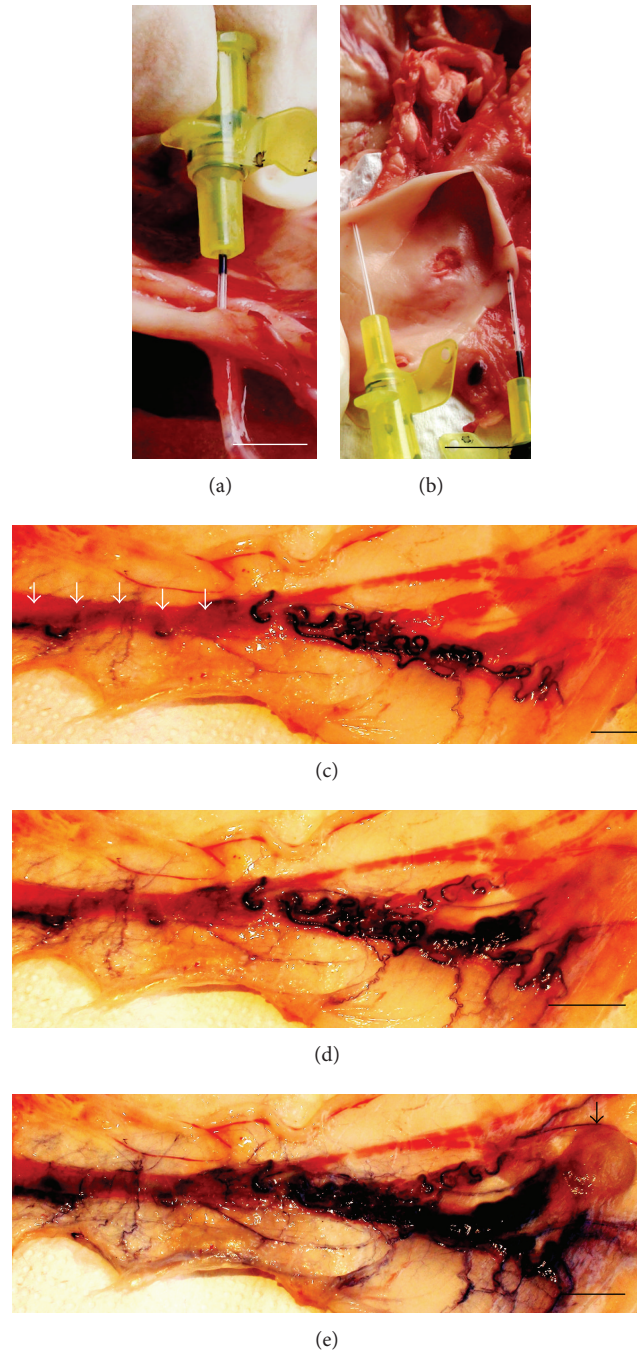


FIGURE 2: *In vitro* perfusion of ovine intact ovary with freezing medium. ((a), (b)) Aorta with arteria ovarica (24 G catheters inside) and ((c), (d), and (e)) perfusion with freezing medium: 3 min (c), 5 min (d), and 10 min (e) elapsed from the beginning of perfusion, vena ovarica (white arrows), ovary (black arrow). Freezing medium includes Leibovitz L-15 medium + 100 IU/mL heparin + 5% bovine calf serum + 6% dimethyl sulfoxide + 6% ethylene glycol + 0.15 M sucrose + Indian ink. Bar = 1 cm.

blood cells in vessels: to expel these cells from vessels before the cooling of ovarian tissue.

We chose the freezing medium for perfusion of ovaries because this freezing medium includes DMSO, liquid with very high rate of permeability. Presence of DMSO and heparin in the perfusion medium increases the ability of the freezing medium to evacuate blood cells from vessels before the freezing. Additionally, the saturation of ovarian tissue by

permeable cryoprotectants was the second aim of perfusion of this tissue with freezing medium.

It was recognized that the presence of blood vessels is a very important ingredient for the successful ovarian tissue transplantation, and the establishment of the blood supplies is crucial for the survival of ovarian follicles [8]. It was demonstrated that transplanted immature rat ovaries become profusely revascularised within 48 h after autotransplantation

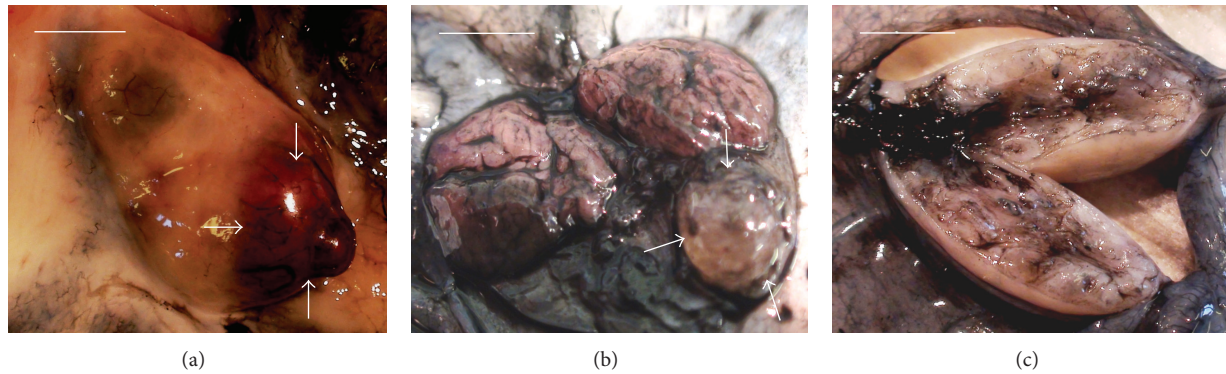


FIGURE 3: End of perfusion of ovine intact ovary with vascular pedicle with freezing medium. ((a), (b)) Ovary after successful perfusion (~95% of tissues excluding yellow body) and (c) ovary after unsuccessful perfusion (~10% of tissues), yellow body (arrows). Note the unsuccessful perfusion of yellow body. Freezing medium includes Leibovitz L-15 medium + 100 IU/mL heparin + 5% bovine calf serum + 6% dimethyl sulfoxide + 6% ethylene glycol + 0.15 M sucrose + Indian ink. Bar = 0.5 cm.

[8]. In some opinions, in the cortex, development of primordial follicles is fully dependent on stromal vessels [9]. Prior to revascularisation, implants are vulnerable to ischemia, which is the primary obstruction to the survival of tissue after transplantation. Such damage can lead to a 30 to 70% decrease in graft size accompanied with fibrotic changes [10]. The hypoxia observed during the first 5 days after grafting and ischemic damage occurring during this period could induce primordial follicle loss [11–13] and disorders of follicular activation [14, 15].

In contrast with transplantation of cortex and transplantation of intact ovaries with pedicles, adequate perfusion can indirectly eliminate ischemia after postthawing transplantation.

During cryopreservation of whole ovaries with postthawing retransplantation through vascular anastomosis, the problem of neovascularisation will be solved “automatically,” because “old” capillaries begin to function just after connection of *arteria ovarica* with the circulatory system.

The data about the successful transplantation of human intact ovary with its vascular pedicle after cryopreservation are absent. However, cryopreservation of whole ovaries with vascular anastomosis with postthawing retransplantation can be viewed as a promising strategy for cancer patients. Some reports have described fresh (nonfrozen) whole ovaries with vascular anastomosis in human [16–18], and one pregnancy was observed [19].

3.2. Our Hypothetical Supposition for Performing of These Experiments. The following hypothetical supposition was the ground for performing of our comparative experiments. We did presuppose that blood which goes through the capillaries of ovarian tissue will be removed from these capillaries more effectively *in vivo* than *in vitro*. We guessed that, if we connect *arteria ovarica* through perfusor with cryopreservation medium, it will create the stable increasing pressure of this medium, and then blood slowly will be replaced by freezing medium. After isolation of *arteria ovarica* and *vena ovarica* from circulating system, we will obtain ovary that

will be free from blood and ready for freezing. Likewise, we have presupposed that we will perform our perfusion manipulations *in vivo* when normal physiological negative pressure in the ovary from site of *vena ovarica* is observed. Theoretically, by *in vivo* perfusion of ovary the herd can play the role of additional pump for perfusion solution: this fact will help perfusion medium to penetrate through the capillaries of the ovary.

3.3. Cryopreservation of Whole Animal Ovaries at Present. The problem of cryopreservation of intact ovary with its vascular pedicle in animals is likewise far from solving.

Despite numerous attempts and the evidence of restoration of long-time follicular development [5, 6, 20–29], only three births after retransplantation of frozen ovine ovaries were noted.

The important role of perfusion of whole ewe ovaries by cryoprotective medium was recently demonstrated [4]. An observational study of 360 ewe ovaries stained by *in vitro* perfusion with qualitative marker of tissue blood supply was performed. A logistic regression model was established to identify the genes associated with incomplete ovary staining. Whole ewe ovaries with their vascular pedicles were perfused at 0.35 mL/min for 2 h at 39°C under 19 experimental conditions. The pedicles were removed and the ovaries cut and photographed. The unstained area of the slice surface was measured. It was noted that unstained areas were observed in 64.4% of the ovaries. Multivariate analysis found that incomplete ovary staining was independently associated with the lower experimenter experience, smaller ovary slice surface area, and the presence of a *corpus luteum*. The presence of unstained areas was independent of experimental conditions. The effectiveness of ovarian perfusion decreased from 83 to 60% [4]. The formulated conclusion of these experiments is that blood-supply impairments that result in incomplete perfusion might adversely affect outcomes after whole ovary cryopreservation. Improved perfusion techniques should enhance success [4].

In experiments of Gerritse et al. [30] the Indian ink perfusion studies were performed on bovine intact ovaries. The authors noticed that especially the larger vessels were well perfused, whereas the smaller vessels and the capillaries were less well perfused. The authors assume that the relatively smaller molecular weights of cryoprotective agents such as dimethyl sulfoxide and sucrose imply that they would be less subject to molecular filtering mechanisms of endothelial cells and basal membranes [30].

The presence of cryoprotectants (especially nonpermeable cryoprotectant sucrose) and bovine calf serum in the perfusion solution can explain that for the successful perfusion of small blood vessels and especially capillaries we need the speed of the perfusion medium slower than in experiments of Gerritse et al. [30] (2.5 mL/min).

3.4. DMSO in Perfusion Solution. Our freezing medium includes 6% DMSO and we used this cryoprotectant at 35–37°C.

What could happen when ovarian tissue will be perfused and later frozen with DMSO? Is it dangerous? To answer this question, we can image another object, which is more cryosensitive than ovarian tissue: human oocytes.

Earlier we performed a series of experiments to look into the role of DMSO during the cryopreservation by direct plunging into liquid nitrogen (vitrification) of human GV-oocytes. In one experiment, we studied the possibility of using a vitrification solution that was completely free of DMSO [31] because DMSO reportedly affects the organization of microfilaments in mouse oocytes [32] and induces chromosomal abnormalities (i.e., increases in the rates of degeneration and polyploid embryos) after cryopreservation of mouse oocytes in the presence of DMSO [33]. However, in our experiments, the absence of DMSO in the vitrification medium caused a significant decrease in maturation rates. On the other hand, a 5-minute contact of oocytes with DMSO at 37°C was enough to cause spontaneous oocyte activation and parthenogenetic development [31]. However, we have found the following solution of the problem. The protocol, which we recommend for use in medical practice, is a compromise between the presence of DMSO in vitrification medium and parthenogenetic activation. On the basis of our results, we propose (1) short (1-minute) contact with DMSO, which is only included in the last solution of vitrification medium, and (2) contact with DMSO at room temperature, which can theoretically decrease the negative effect on oocytes. The high maturation rates and the absence of parthenogenesis are a direct proof that this way of using DMSO (decreasing time and temperature of contact) is suitable for vitrification of human GV-oocytes.

On what ground do we have to say that 6% DMSO in perfusion medium (and later in freezing mediums) is not toxic for ovarian tissue?

Firstly, toxicity of this cryoprotectant is brightly discussed in cases of vitrification of oocytes and embryos, when concentration is up to 20%. Our perfusion and freezing solution includes only 6% DMSO. In medical practice DMSO

in such concentration is brightly using for cryopreservation of a number of different tissues [7, 34–43].

Secondly, the potential toxic effect of DMSO can be compensated by detergent properties of DMSO, due to the presence of which the permeability of the perfusion medium through small capillaries will be increased.

4. Conclusions

In conclusion, *in vitro* perfusion of ovine whole ovaries with vascular pedicle by freezing medium is more effective than this manipulation performed *in vivo*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Effect of Hyaluronan on Developmental Competence and Quality of Oocytes and Obtained Blastocysts from *In Vitro* Maturation of Bovine Oocytes

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The objective of the present study was to evaluate the effect of hyaluronan (HA) during IVM on meiotic maturation, embryonic development, and the quality of oocytes, granulosa cells (GC), and obtained blastocysts. COCs were matured *in vitro* in control medium and medium with additional 0.035% or 0.07% of exogenous HA. The meiotic maturity did not differ between the analysed groups. The best rate and the highest quality of obtained blastocysts were observed when 0.07% HA was used. A highly significant difference ($P < 0.001$) was noted in the mean number of apoptotic nuclei per blastocyst and in the DCI between the 0.07% HA and the control blastocysts ($P < 0.01$). Our results suggest that addition of 0.035% HA and 0.07% HA to oocyte maturation media does not affect oocyte nuclear maturation and DNA fragmentation. However, the addition of 0.07% HA during IVM decreases the level of blastocysts DNA fragmentation. Finally, our results suggest that it may be risky to increase the HA concentration during IVM above 0.07% as we found significantly higher *Bax* mRNA expression levels in GC cultured with 0.07% HA. The final concentration of HA being supplemented to oocyte maturation media is critical for the success of the IVP procedure.

1. Introduction

The large discrepancy between the number of oocytes undergoing *in vitro* fertilisation and the number of embryos developing to the blastocyst stage (approximately 30–40%) calls for further research to improve the oocyte maturation medium in order to mimic the *in vivo* maturation conditions. A few studies have focused their attention on the supplementation of *in vitro* embryo culture media with hyaluronan (HA) due to its biochemical properties [1–3]. Important biological functions are not properly regulated during *in vitro* culture due to the two-dimensional nature of the culture. Hyaluronan forms a concentration dependent, three-dimensional, jelly-like network. A three-dimensional network improves the interaction between cellular receptors and the molecules in the external environment by improving the binding of molecules such as polypeptide growth factors that play an important

role during early embryonic development [4]. Hyaluronan is a major component of the cumulus-oocyte complex (COCs) and is synthesised and secreted by granulosa cells under the stimulation of LH and FSH [5]. The insufficient interactions of hyaluronan and its receptor CD44 during *in vitro* maturation may decrease the capacity of fertilization and development of oocytes matured *in vitro* [6]. The resumption of meiosis in mares, pigs, and rats is associated with decreased levels of connexin 43 (Cx43) [7]. Yokoo et al. [8] revealed that the interaction of HA with the CD44 receptor in granulosa cells during cumulus expansion lowers the levels of Cx43. As a result of this process, the transport of cAMP between cells and the oocyte is blocked. A decrease in the concentration of cAMP in oocytes is a signal for the activation of MPF, which is responsible for the resumption of meiosis (GVBD) [8]. In addition, HA participates in the process of sperm capacitation [9], determines the proper course of fertilisation, and affects

the growth potential of early embryos [10]. Many cellular processes are regulated by the interaction between HA and its surface receptors, such as CD44, RHAMM (receptor for HA mediated motility), and ICAM-1 (intercellular adhesion molecule 1) [11, 12]. These processes include the maintenance of tissue homeostasis [13], cell adhesion and migration [14, 15], cell proliferation and differentiation, and the inhibition of apoptosis and immune response [16, 17].

The objective of the present study was to evaluate the HA supplementation effect on meiotic maturation at two concentrations within *in vitro* maturation medium. Additionally, embryonic development after *in vitro* fertilisation as well as the quality of bovine oocytes, granulosa cells that served as coculture during IVM, and obtained blastocysts will be evaluated. The results of our previous experiments [18] have indicated that the supplementation of IVM with HA did not increase the nuclear maturity of oocytes. Moreover, our previous results have indicated that the 0.035% and 0.07% HA concentrations present opposing effects on IVM. The meiotic maturity of the 0.07% HA treated oocytes was the lowest [18], inclining us to apply them in this experiment. Therefore, in the present experiment, we TUNEL analysed oocytes in two experimental groups. Moreover, blastocysts obtained after fertilisation of HA treated COCs provided data on their developmental competence.

2. Materials and Methods

Immature oocytes were isolated from ovaries obtained from heifers and cows from the slaughterhouse, which were delivered in a warm flask to the laboratory within 2-3 hours after slaughter.

2.1. Chemicals and Supplies. The reagents used in the presented experiments were purchased from Sigma-Aldrich, Poland, unless otherwise indicated.

2.2. Experimental Design. The research was performed in two directions. First, we evaluated the effect of two concentrations of HA supplementation on meiotic maturation and embryonic development after *in vitro* fertilisation. Second, we evaluated the possible negative impact of HA on the oocytes, the blastocysts, and granulosa cells. TUNEL staining and the estimation of apoptotic genes transcripts expression by real-time PCR were employed as quality markers.

2.3. Recovery and Selection of Bovine Oocytes. The detailed description of procedure is provided in Opiela et al. [18]. Cumulus-oocyte complexes (COCs) were retrieved from ovaries of slaughtered cows by aspiration of follicular fluid of ovarian follicles. For this purpose, a syringe and needle were used. Follicular fluid was collected into tubes (Corning, 12 mL) and left for 15–30 minutes at room temperature to sediment. The supernatant was then collected, and the remaining sediment was transferred into a holding medium (TCM 199—Earle's salt with 25 mM of HEPES containing 10% foetal calf serum). Next, COCs were recovered under stereomicroscope. Before placing COCs into the *in vitro*

maturation, they were washed three times for selection. Degenerated oocytes and those without compact cumulus cells were discarded.

2.4. In Vitro Maturation in Coculture with Granulosa Cells. For the culture of COCs the commercial medium TCM-199 buffered with sodium bicarbonate and supplemented with L-glutamine was used. Selected COCs were cultured for 22 hours in 2 mL of medium supplemented with 20% estrus cow serum (ECS, heat inactivated-prepared in our laboratory) and an additional 3 to 5×10^6 granulosa cells/mL (GC). Between 30 and 40 COCs were used per culture dish. Granulosa cells were obtained from follicles with a diameter of 4–6 mm without atretic changes and containing oocytes with normal morphological appearance. The obtained granulosa cells were suspended in 7 mL of *in vitro* maturation medium without exogenous HA. After carefully mixing the medium with GC, the suspension was divided between 3 culture dishes, for 2 mL each. *In vitro* maturation was performed with different concentrations of 1% high molecular weight HA (Croma Pharma GmbH, Austria), with 75 μ L/2 mL medium (a final concentration of HA 0.035%) and 150 μ L/2 mL medium (a final concentration of 0.07%). As a control, we used COCs matured only in maturation medium and cocultured with granulosa cells [18]. This approach was provided the same way to experimental conditions in all of the groups, as the estrus cow serum may contain HA and HA is also synthesised and secreted by granulosa cells in undefined and variable amount. Moreover, the same ESC was used in all IVM media during the whole experiment and each repetition of IVM/IVF/IVC was performed in three simultaneous experimental groups. Oocytes matured for 22 h at 38.5°C under 5% CO₂ in air.

2.5. Effect of HA on Meiotic Maturity and Chromatin Fragmentation in Oocytes

2.5.1. TUNEL. The detailed description of procedure is provided in Opiela et al. [18]. TUNEL analysis was performed using a Deadend Fluorometric TUNEL System (Promega, Poland).

Fixed denuded oocytes were first incubated with 0.2% Triton X-100 solution for 5 minutes, then a reaction mixture consisting of equilibration buffer, the mixture of nucleotides and TdT enzyme for 1 h at maximum humidity, and lastly a solution of 2 \times SSC for 15 mins. After the performed incubations, the oocytes were washed three times in PBS/PVP for at least 5 minutes. Finally, the oocytes were placed in a drop of VECTASHIELD + DAPI solution. Oocytes that were treated with UV light for 30 mins immediately after maturation were used as a positive control. As a negative control, oocytes placed in the reaction mixture lacking the rTdT enzyme were used. Oocytes were analysed under a fluorescent microscope. To visualise the stages of meiosis and DNA fragmentation of oocytes, a filter of >460 nm was used for the blue fluorescence of DAPI stained cells and a filter with a wavelength of 520 \pm 20 nm was used for the green fluorescence of apoptotic cells, which incorporated into the nucleus with a fluorescein-conjugated dUTP.

2.5.2. Estimation of Meiotic Maturity. We have applied similar criteria to those of Warzych et al. [19]. In cases of strong fluorescent/DAPI signals from the nuclei and the polar body, meiotic stage classification (GV, MI, AI, TI, MII) was performed. A detailed description of assessment criteria is also available in Opiela et al. [18]. Briefly, the germinal vesicle (GV) was characterised by varying degrees of chromatin condensation, metaphase of first meiotic division (MI) was characterised by chromosomes arranged as a group of separated bivalents, and metaphase second (MII) was characterised by the presence of haploid set of chromosomes and chromosomes forming the first polar body (PBI). Meiotic maturity was counted as the ratio of oocytes in metaphase II stage to the total number of analysed oocytes.

2.5.3. Estimation of Oocytes Apoptotic Index DCI. TUNEL positive oocytes indicated fragmented DNA by a strong fluorescent/FITC signal from the nuclei or polar body. As a control for the experiment, the COCs were subjected to the UV (30 mins. at room temperature in the M199 medium containing HEPES supplemented with 5% FCS). This generated “damage” to the DNA, which would show a positive TUNEL signal and indicate that the TUNEL reaction works. The death cell index (DCI) was expressed as a ratio of apoptotic oocytes exhibiting a strong fluorescent/FITC signal to all analysed oocytes from each group [18, 19].

2.6. Effect of HA on the Developmental Competence of Bovine Oocytes

2.6.1. In Vitro Fertilisation. IVF was performed according to our standard protocol that was previously described [20, 21]. Briefly, after 22–24 hrs of maturation, the selected oocytes were fertilised using frozen semen from single bull. The motile sperm were selected by centrifugation (300 g at room temp.) on a discontinuous (1 mL 45% over 1 mL 90%) Percoll (Pharmacia, Sweden). After washing, the sperm were suspended in the fertilisation medium containing 10 µg/mL of heparin and a mixture of penicillamine (20 µM), hypotaurine (10 µM), and epinephrine (1 µM) at a concentration of $1\text{--}2 \times 10^6$ spermatozoa/mL of medium. After washing and partial deprivation of the expanded cumulus, the mature COCs were transferred in groups of 10 into 50 µL of fertilisation medium. Gametes were incubated together for 18 to 21 h at 38.5°C under 5% CO₂ in air [20, 21].

2.6.2. In Vitro Embryo Culture and VERO Cell Preparation. IVC and VERO cell preparation was performed according to our previously described standard protocol [20, 21]. Briefly, at 24 h after fertilisation, the presumptive zygotes were transferred into 50 µL drops of B₂ medium (C.C.D., Paris, France) under mineral oil. The number of cleaved embryos was recorded on Day 2 following fertilisation, and approximately 20 embryos were placed in coculture with Vero cells in 50 µL drops of B₂ medium supplemented with 2.5% FCS, under mineral oil. Medium in culture drops was partially changed (20 µL) at intervals of 48 h. Embryos were maintained in

coculture for 7 to 8 days. At the end of the culture period, the total blastocyst rate and the hatching rate were recorded.

Vero cells were obtained frozen from ECAGC, Salisbury, UK. Cells were seeded at a concentration of 1×10^6 cells in 5 mL of medium per flask (for passages) and 1×10^2 cells in 50 µL of medium per drop (for coculture with the small group of embryos). The medium for coculture, B₂ medium enriched with 2.5% FCS, was changed before the embryos were added and then partially changed every 48 h. The *in vitro* embryo culture was performed at 38.5°C under 5% CO₂ in air.

2.7. Effect of HA on Blastocysts Quality Measured by TUNEL. TUNEL analysis was performed using a Deadend Fluorometric TUNEL System, (Promega, Poland) according to the protocol described in Warzych et al. [19]. The analysis was performed using a fluorescent microscope (Nikon Eclipse E600).

2.7.1. Estimation of Blastocysts Apoptotic Index, DCI. Day 8 blastocysts (middle, late, expanding, and hatched) were subjected to TUNEL analysis. The number of all blastomere nuclei and the number of all apoptotic nuclei were recorded for each embryo. To assess the DCI for the single blastocyst the sum of all apoptotic nuclei detected in the analysed blastocyst was divided by the sum of all nuclei detected in analysed blastocyst and multiplied by 100.

2.8. Effect of HA on Bax and Bcl-2 Transcript Levels in Granulosa Cells Used as Coculture during IVM

2.8.1. RNA Isolation, RT, and Quantitative PCR (qPCR). After IVM, the remaining culture medium of the three experimental groups with cocultured granulosa cells was transferred into Eppendorf tubes for centrifugation (300 ×g). The remaining pellet of cells was washed in PBS (without Mg²⁺ and Ca²⁺) three times. After washing, the cells were resuspended in 10 µL of PBS, snap frozen in liquid nitrogen, and stored at –80°C until use. The samples from the three experimental groups had the same number of cells as during the granulosa-cells coculture setup. The cells obtained from the follicles were equally divided into three culture dishes, the control, HA at 0.035%, and HA at 0.07%. This experiment was repeated three times with the same COCs number for each repetition (40–50 COCs).

Total RNA was isolated as previously described [22]. The relative expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to normalise the marker gene expression in each sample. A One-Step Brilliant II SYBR Green QRT-PCR Master Mix Kit (Stratagene, La Jolla, CA, USA) was used to perform relative quantification of gene expression. Each PCR reaction (total volume of 25 µL) consisted of total RNA (2 ng/µL), 1× of SYBR Green QRT-PCR master mix (contains an optimised RT-PCR buffer, 2.5 nM of MgCl₂, nucleotides (GAUC), SureStart Taq DNA polymerase, SYBR Green and stabilisers), 200 nM each of the forward and reverse primer [21], and 1× of RT/RNase block enzyme mixture. Thermal cycling conditions were as follows: 30 mins at 50°C (for the first-strand synthesis); 10 mins

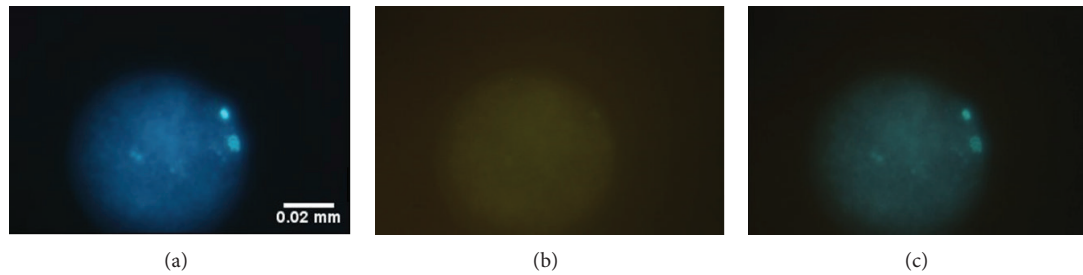


FIGURE 1: Bovine oocyte after TUNEL staining. (a) DAPI staining of chromatin in metaphase II stage and the 1st polar body; (b) absence of green fluorescence after FITC staining of the same oocyte; (c) photos (a) and (b) merged.

at 95°C; 35 cycles of 30 s at 95°C for denaturing; 60 s at 57°C for annealing; and 30 s at 72°C for extension. Experiments were carried out on Mastercycler ep realplex apparatus (Eppendorf, UK Limited, Cambridge). GAPDH was used as an endogenous standard. The results for individual target genes were normalised according to the relative concentration of the endogenous standard. Each reaction was run in triplicate and the obtained results were averaged. The results for the GC HA 0.035% and GC HA 0.07% were compared with control granulosa cells which served as a calibrator. The 2- $^{-\Delta\Delta Ct}$ method was used for calculating the relative quantification.

2.9. Statistical Analysis. Differences in TUNEL results from oocytes and developmental competence of fertilised oocytes were assessed using chi-square test (χ^2). Differences in *Bax* and *Bcl-2* transcripts level were assessed using ANOVA followed by Tuckey's post hoc test. The same test was performed in case of TUNEL results from blastocysts regarding all nuclei, apoptotic nuclei, and DCI. In all tests differences with a probability value of 0.05 or less were considered significant.

3. Results

3.1. Effect of HA on the Meiotic Maturity and Chromatin Fragmentation of Oocytes. To evaluate the HA impact on oocytes after *in vitro* culture, the estimation of meiosis stage and the level of DNA fragmentation was performed by TUNEL staining (Figure 1). The TUNEL staining was analysed from 428 oocytes matured under three media conditions in a volume of 2 mL in the presence of granulosa cells. Of the oocytes evaluated, 151 oocytes in the control medium without HA matured, 133 of analysed oocytes matured in medium with 0.035% HA, and 144 oocytes matured in medium with 0.07% HA. The highest meiotic maturation (70.2%) was observed in oocytes cultured in the control medium, and the lowest maturation was in medium supplemented with 0.07% HA (63.1%) (Table 1). In medium supplemented with 0.035% HA, the meiotic maturity was almost 67.7% (Table 1). The obtained meiotic maturity did not differ between the analysed groups. We did not find any signs of DNA fragmentation in any of oocytes within the three analysed groups (Figure 1). This experiment showed that HA supplementation did not have a detrimental impact on oocyte chromatin integrity.

TABLE 1: Effect of HA on oocytes meiotic maturity and chromatin fragmentation (replicates = 4).

Medium	Oocytes <i>n</i>	Maturation stage		
		MII <i>n</i> (%) (mean \pm S.D)	MI <i>n</i>	GV <i>n</i>
Control	151	106 (70.2%) (26.5 \pm 4.36)	42	3
0.035% HA	133	90 (67.7%) (22.5 \pm 5.45)	37	6
0.07% HA	144	91 (63.1%) (22.75 \pm 5.91)	48	5

Values within column do not differ significantly (test χ^2).
n: number; MII: metaphase II; MI: metaphase I; GV: germinal vesicle.

TABLE 2: The effect of HA supplementation on developmental competence of bovine oocytes (5 replicates) (mean \pm S.D.).

Medium	<i>n</i> of oocytes	Cleaved <i>n</i> (%) (mean \pm S.D.)	Blastocyst <i>n</i> (%) (mean \pm S.D.)
0.035% HA	174	114 (65.5) (22.8 \pm 5.26) ^{A,a}	22 (13) (4.4 \pm 3.04) ^a
0.07% HA	169	124 (73.4) (24.8 \pm 7.05) ^b	39 (23.1) (7.8 \pm 2.38) ^b
Control	186	148 (79.5) (29.6 \pm 6.66) ^B	33 (17.7) (6.6 \pm 4.97)

Values within column with different superscripts differ significantly: ^{a,b}*P* < 0.05; ^{A,B}*P* < 0.001 (test χ^2). *n*: number.

3.2. Effect of HA on the Developmental Competence of Bovine Oocytes. Although the number of cleaved eggs was significantly lower when gametes were matured in the presence of HA (66% and 73% for 0.035 and 0.07% HA, resp.), the number of obtained blastocysts was the highest when 0.07% HA was used. However, there was no significant difference between this group of oocytes and control (Table 2). The lowest number of cleaved eggs was observed for oocytes matured with 0.035% HA in relation to the control (*P* < 0.001) and medium supplemented with 0.07% HA (*P* < 0.05). The lowest number of obtained blastocysts was observed for oocytes matured with 0.035% HA relative to the medium with 0.07% HA (*P* < 0.05; Table 2).

TABLE 3: The effect of HA supplementation on blastocyst quality measured by DNA fragmentation of blastomeres.

Medium	<i>n</i> blastocysts	Mean number of nuclei per blastocyst \pm S.D.	Mean number of apoptotic nuclei per blastocyst \pm S.D.	Mean DCI per blastocyst \pm S.D.
0.035% HA	20	84.4 \pm 33.82 ^a	9.1 \pm 7.64 ^a	13.91 \pm 18.2
0.07% HA	29	86.83 \pm 38.95 ^a	6.45 \pm 3.28 ^{a,A}	8.89 \pm 5.35 ^C
Control	27	81.67 \pm 27.65 ^a	14.37 \pm 7.15 ^{b,B}	20.34 \pm 13.5 ^D

^{a,b} $P < 0.05$, ^{A,B} $P < 0.001$, ^{C,D} $P < 0.01$ ANOVA followed by Tuckey's post hoc test.

n: number; DCI: death cell index.

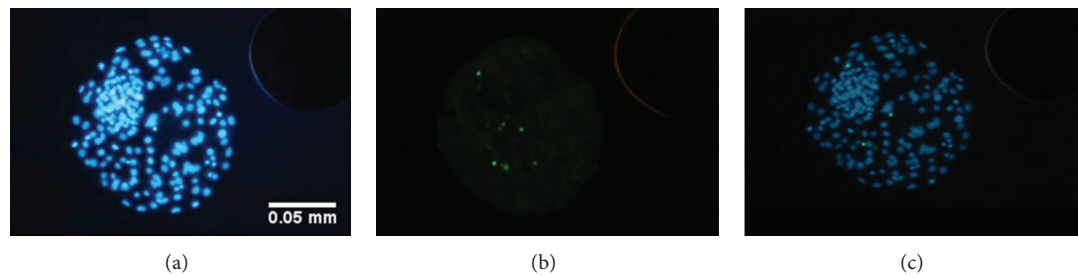


FIGURE 2: Bovine blastocyst after TUNEL staining. (a) DAPI staining of all blastomere nuclei; (b) green FITC staining of apoptotic nuclei; (c) photos (a) and (b) merged.

3.3. Effect of HA on Blastocysts Quality Measured by TUNEL.

The quality of blastocysts developed from oocytes matured with 0.07% HA was the highest. A highly significant difference ($P < 0.001$) was noted in the mean number of apoptotic nuclei per blastocyst and a highly significant difference ($P < 0.01$) was noted in the DCI between the 0.07% HA blastocysts and the control blastocysts (Table 3, Figure 2). The control blastocysts had the highest mean number of apoptotic nuclei per blastocyst when compared to blastocysts developed from oocytes cultured with 0.07% HA ($P < 0.001$) and 0.035% HA ($P < 0.05$) (Table 3). There were no significant differences between the mean number of nuclei per blastocyst in all analysed groups of blastocysts (Table 3, Figure 2).

3.4. Effect of HA on Bax and Bcl-2 Transcript Levels in Granulosa Cells. To better analyse the possible negative impact of applied concentrations of HA for IVM conditions, we estimated the relative Bax and Bcl-2 transcript levels in cocultured granulosa cells. We found significantly higher Bax mRNA expression in GC cultured with 0.07% HA as compared to the control ($P < 0.05$) and from 0.035% HA GC ($P < 0.01$) (Figure 3). No significant differences were noted in the relative Bcl-2 expression between the analysed groups of GC (Figure 4).

4. Discussion

The present study evaluated the effect of exogenous HA supplementation of *in vitro* maturation medium. Changes in meiotic maturation, embryonic development after *in vitro* fertilisation, the quality of bovine oocytes and IVM cocultured granulosa cells, and the quality of obtained blastocysts were measured. Yokoo et al. [6] showed that after culture of oocytes in the presence of anti-CD44 antibody, the suppression of MPF and an inability to induce GVBD were observed.

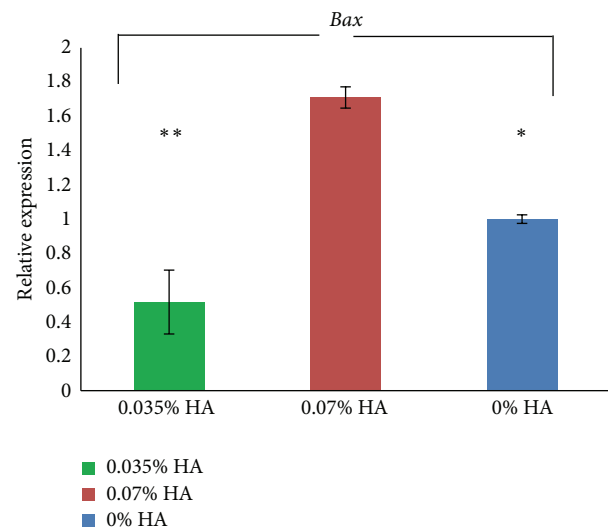


FIGURE 3: Relative mRNA expressions (mean \pm SD) for Bax in granulosa cells which served as coculture during IVM of oocytes cultured with addition of 0.035% HA, 0.07% HA, and control. The significant differences were noted **($P < 0.01$), *($P < 0.05$) (ANOVA followed by Tuckey's post hoc test)

The interaction between hyaluronan and its main receptor CD44 results in closing the gap junctions and the cessation cAMP transport from granulosa cells to the ooplasm, which in turn triggers the activation of MPF and resumption of meiosis [6]. Based on these data, it was assumed that the addition of exogenous hyaluronan could increase the percentage of oocytes reaching the metaphase II stage. So far, there is only one paper reporting on the role of exogenous HA supplementation during bovine *in vitro* oocyte maturation and further embryo development [23]. The authors

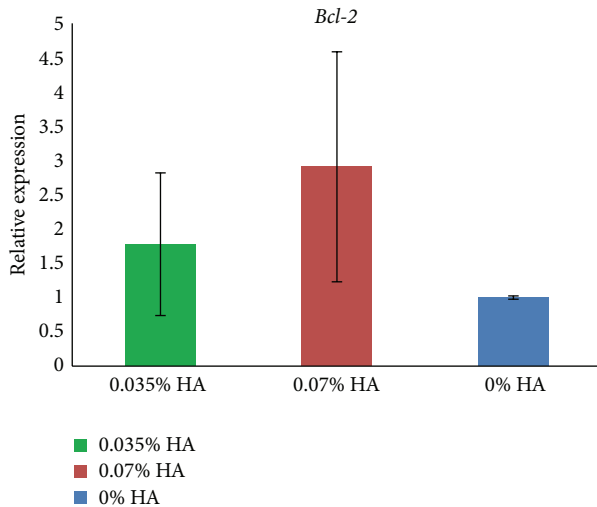


FIGURE 4: Relative mRNA expressions (mean \pm SD) for *Bcl-2* in granulosa cells which served as coculture during IVM of oocytes. The expression of levels of *Bcl-2* in granulosa cells was measured after the addition of 0.035% HA, 0.07% HA, and at control levels. No significant differences were noted (ANOVA followed by Tuckey's post hoc test).

matured oocytes in the presence or absence of HA, Hyal-2 (Hyaluronidase enzyme), or 4-methylumbelliferone (4-MU), an HA synthesis inhibitor [23]. When we started our research, no information was available regarding the HA concentration for successful bovine oocyte maturation and blastocyst development after fertilisation. During preparation of this paper, Marei et al. [23] published their paper reporting the effect of IVM supplementation with HA at 0.1, 0.5 and 1 mg/mL on oocyte maturation but only reported the effect of 0.5 mg/mL of HA on embryo development and quality. Their concentration of 0.5 mg/mL is the mean concentration of the 0.75 mg/mL and 0.375 mg/mL applied by us. Marei et al. [23] have shown that the addition of 0.5 mg/mL of HA to oocyte maturation media did not affect cumulus cell expansion or oocyte nuclear maturation.

Our results support their observations, as we did not find any significant difference between the analysed groups. However, based on the rates of mature oocytes in three experimental oocytes groups, it is noticeable that the highest meiotic maturation was observed in oocytes cultured in control medium and the lowest in medium supplemented with 0.07% HA. Based on data regarding HA synthesis and reduction [24, 25], the authors [23] suggested that a low concentration of HA is required for signalling mechanisms contributing to oocyte nuclear maturation and that no effect can be seen on nuclear maturation if HA production is not markedly inhibited. In contrast, high HA concentration could be harmful [26]. Our results seem to support this assumption. We also did not find any signs of DNA fragmentation in any of oocytes within the three analysed groups, which proves that HA does not have a detrimental impact on DNA fragmentation.

Although the number of cleaved eggs was significantly lower when gametes were matured in presence of HA, the number of obtained blastocysts was the highest when 0.07% HA was used. However, there was no significant difference between this group of oocytes and control. These data make determining the effect of HA difficult. Looking at the higher percentage of developed blastocysts in the 0.07% HA group as compared to the remaining two groups, it can be assumed that a significant difference could be noted when larger populations of oocytes are fertilised. Still, it is quite an interesting observation that a statistically lower number of obtained blastocysts developed from oocytes matured in 0.035% HA as compared to 0.07% HA. It seems that the applied HA concentration is the key to success. We assume that there is a certain threshold level of HA after which the positive effect is reached.

This explanation may be confirmed by another observation regarding blastocyst quality as measured by TUNEL staining. Interestingly, we found that blastocysts developed from oocytes matured with 0.07% HA had the best quality. A high significant difference in DCI was noted between these blastocysts and the control ($P < 0.001$). The number of apoptotic nuclei in blastocysts developed from oocytes cultured in the presence of HA (both concentrations) was statistically lower when compared to the control. Based on these results, we can conclude that supplementation of exogenous HA during oocyte IVM improves the quality of developed embryos.

To better analyse the possible negative impact of applied concentrations of HA for IVM conditions, we estimated the relative *Bax* and *Bcl-2* transcript levels in cocultured granulosa cells. We found significantly higher *Bax* mRNA expression levels in GC cultured with 0.07% HA compared to controls ($P < 0.05$) and GC cultured with 0.035% HA ($P < 0.01$). The higher *Bax* expression can mean that HA acted as stimulus initiating apoptosis. Although no significant differences were noted in *Bcl-2* relative expression between analysed groups of GC, the highest *Bcl-2* expression was observed in GC cultured with 0.07% HA. These data show a typical interplay between the pro- and antiapoptotic genes and proteins in the apoptotic pathway [21]. The incidence of apoptosis in granulosa cells/cumulus cells is treated as a good indicator of oocyte developmental competence [27–29], as they regulate the maturation of nucleus and cytoplasm in oocytes. Moreover, some authors claim that signs of early atresia in oocytes or cumulus cells translate into better developmental competence [21, 29, 30]. Taking the above mentioned points into account, it may be risky to increase the concentration of HA during IVM above a final concentration of 0.07%. Because the cleavage rate of oocytes cultured with 0.07% HA was the lowest and the *Bax* expression of GC was the highest, these culture conditions may not be optimal for the oocytes, as some are eliminated; however, the remaining oocytes are of better quality, which is reflected in the increased number of developed blastocysts and improved quality (lowest DCI).

In conclusion, our results suggest that addition of 0.07% HA to the *in vitro* maturation medium significantly decreases the level of blastocyst DNA fragmentation. The final

concentration of HA being supplemented to oocyte maturation media is critical for the success of the IVP procedure. Moreover, there is a noticeable positive impact on the number of developed blastocysts with 0.07% HA during IVF.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Clinical Study

Evaluation of Granulocyte Colony-Stimulating Factor Effects on Treatment-Resistant Thin Endometrium in Women Undergoing *In Vitro* Fertilization

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The aim of the study was to assess the granulocyte colony-stimulating factor (G-CSF) effects on unresponsive thin (<7 mm) endometrium in women undergoing *in vitro* fertilization (IVF). We included thirty-seven subjects who had thin unresponsive endometrium on the day of triggering ovulation. These patients also failed to achieve an adequate endometrial thickness in at least one of their previous IVF cycles. In all the subjects at the time of infusion of G-CSF, endometrial thickness was 6.74 ± 1.75 mm, and, after infusion, it increased significantly to 8.42 ± 1.73 mm. When we divided the group into two subgroups according to whether the examined women conceived, we showed that the endometrium expanded significantly from 6.86 ± 1.65 to 8.80 ± 1.14 mm in the first group (who conceived) and from 6.71 ± 1.80 to 8.33 ± 1.85 mm in the second, respectively. There were no significant differences between the two subgroups in respect to the endometrial thickness both before and after G-CSF infusion. The clinical pregnancy rate was 18.9%. We concluded that the infusion of G-CSF leads to the improvement of endometrium thickness after 72 hours.

1. Introduction

Many factors could have impact on the *in vitro* fertilization-embryo transfer IVF-ET success. The main independent variables are: the age of women, antimüllerian hormone (AMH) concentrations, number of embryos transferred and their quality [1]. It was also demonstrated that endometrial thickness <7 mm negatively affected pregnancy rate [2, 3]. Moreover, Sharkey showed that immunological mechanisms in the endometrium are very important and crucial in the implantation process [4]. Some investigators demonstrated that the growth factors, hormones, and cytokines, which are produced by decidual cells, are involved in the implantation process [5]. Preliminary studies demonstrated that G-CSF

stimulated neutrophilic granulocyte proliferation and differentiation, acted on macrophages of decidual cells, and finally affected the implantation [6, 7]. What is more, known and reported immune effects of G-CSF are recruitment of dendritic cells, promoting Th-2 cytokine secretion, activating T regulatory cells, and also stimulation of various proangiogenic effects [7, 8]. On the other hand, the receptor for G-CSF is expressed by the trophoblastic cells and by human luteinized granulosa cells [9, 10]. It was also stated that G-CSF prevented repeated miscarriages and implantation failures [11, 12]. In the last two years, Gleicher et al. presented two clinical studies with limited number of participants regarding the usefulness of G-CSF treatment in endometrium expansion in women who had previously cancelled cycles because of

the unresponsive endometrium [13, 14]. Taking into account all these data, the aim of the study was to examine G-CSF effects on unresponsive thin (<7 mm) endometrium in women undergoing IVF.

2. Materials and Methods

We presented a series of 37 patients who have undergone IVF procedure.

The inclusion criteria were as follows:

- (a) women aged 18–45 years,
- (b) previously cancelled at least one cycle because of thin unresponsive endometrium (<7 mm) during IVF programs,
- (c) inadequate thin endometrium (<7 mm) on the day of hCG injection,
- (d) the lack of contraindications for G-CSF treatment (sickle cell disease, chronic neutropenia, known past or present malignancy, renal insufficiency, upper respiratory infection, pneumonia, and congenital fructose intolerance),
- (e) personal agreement for such still experimental therapy,
- (f) no prenatal genetic screening,
- (g) no Asherman's syndrome, fibroids, and polyps in diagnostic hysteroscopy.

The study protocol was approved by the Institutional Review Board of Varmia and Masuria, Olsztyn, Poland, and written informed consent was given by each participating women.

The primary end point was the endometrial thickness measured in transvaginal sonography. The second point was clinical pregnancy after embryo transfer. Identification of an intrauterine gestational sac by transvaginal ultrasonography together with an increasing serum β -hCG constituted a clinical state of pregnancy. All included women had previously long agonist protocol in all cases.

During the study, all patients received oral contraceptive pill (OCP) starting on days 2–5 of spontaneous menses of the cycle prior to the treatment cycle. The OCP contained 0.03 mg ethinyl estradiol (E2) and 0.15 mg desogestrel Ovulastan (Polfa, Poland). OCPs were taken daily for 21 days. Patients were administered s.c. GnRH agonist 0.1 mg gonapeptyl (Ferring, The Netherlands) daily. The agonist was started 4–5 days before discontinuation of the OCP. When desensitization was achieved as evidenced by plasma E2 levels of <50 pg/mL [15], daily s.c. injection of highly purified menotropin (Menopur, Ferring, The Netherlands) was commenced. When at least two follicles reached a minimum of 17 mm diameter, 5000 U of hCG (Choragon Ferring, The Netherlands) was applied. Transvaginal pick-up was performed 36 h hours after hCG administration under transvaginal sonography.

The infusion of G-CSF was made according to Gleicher et al.'s procedure with full bladder before transfer [14]. Frydman

catheter was introduced to the uterine cavity. We infused under ultrasound guidance 30 mL (300 mg/1 mL) of G-CSF (Neupogen, Filgrastim, Amgen Inc., Thousand Oaks, CA, USA).

Endometrium was reassessed after 72 hours. If it expanded, all transfers were performed by two doctors. When endometrium was below 7 mm after G-CSF, women could choose two options: to have blastocyst transfer, despite inadequate endometrium, or to cancel the cycle. In that case, embryos were frozen. The number of embryos transferred varied from one to three.

3. Statistical Analysis

Continuous variables were presented as mean \pm SD; categorical variables were presented by ratio. Differences between dependent variables (before and after) were checked by paired *t*-test. Differences between independent variables were checked by *t*-test. $P < 0.05$ was considered statistically significant. The statistical package STATISTICA (data analysis software system), version 10.0 (StatSoft Inc., Tulsa, OK; <http://www.statsoft.com/>), was used for data analysis.

4. Results

The baseline characteristics of participants are shown in Table 1. Table 2 presents endometrial thickness in women before and after infusion of G-CSF who had IVF-ET. In all the subjects at the time of infusion of G-CSF, endometrial thickness was 6.74 ± 1.75 mm, and, after infusion, it increased to 8.42 ± 1.73 mm ($P < 0.001$). When we divided the group into two subgroups according to whether they conceived, we showed that the endometrium increased from 6.86 ± 1.65 to 8.80 ± 1.14 mm in the first one and from 6.71 ± 1.80 to 8.33 ± 1.85 mm in the second one ($P < 0.001$). There were no significant differences between the two subgroups in respect to the endometrial thickness both before ($P = 0.84$) and after infusion ($P = 0.86$). The clinical pregnancy rate was 18.9%. Seven women conceived and delivered. Four women had one single sac and one single term birth. We recorded three women with two gestational sacs. One of them had preterm single live birth, whereas the second delivered prematurely single birth—the baby had intrauterine death—and the third had term birth of twins. No triplets were recorded.

All the women were supplied with low-dose aspirin, sildenafil citrate (Viagra), or both in the treatment cycle and in previous cycles.

Three out of seven women who conceived after G-CSF were pregnant once before entering the study; one woman was pregnant twice. Eight out of thirty in subgroups who did not conceive were pregnant once, whereas four women twice and one woman three times, respectively.

The mean endometrial thickness in previous failures was $5.75 \text{ mm} \pm 1.0 \text{ mm}$ for all women; when we divided women according to whether they conceived, the mean endometrial thickness was $6.45 \pm 0.38 \text{ mm}$ for those who conceived and $5.95 \pm 0.76 \text{ mm}$ for those who did not conceive, respectively.

TABLE 1: Baseline patient characteristics and IVF cycle characteristics in women with thin endometrium.

Characteristic	All women <i>n</i> = 37	Women who conceived <i>n</i> = 7	Women who did not conceive <i>n</i> = 30
Age (years)	34.68 ± 4.13 (35)	32.14 ± 2.79 (33)	35.32 ± 4.20 (36)
Primary infertility diagnosis	24/37 (64.86%)	4/7 (57.14%)	20/30 (66.67%)
Secondary infertility diagnosis	13/37 (35.14%)	3/7 (42.86%)	10/30 (30.33%)
BMI (kg/m ²)	23.09 ± 2.78 (23.31)	21.89 ± 1.37 (22.15)	23.36 ± 2.97 (23.85)
FSH (mIU/mL)	7.18 ± 1.91 (7.3)	6.87 ± 1.40 (7)	7.31 ± 2.19 (7.60)
AMH (ng/mL)	4.28 ± 3.29 (3.8) 0.1–12.8	6.37 ± 4.17 (4.60) 1–12.8	3.78 ± 2.91 (3.4) 0.1–10.8
Cycles	3.46 ± 2.23 (3.00) 1–11	3.29 ± 1.80 (3.00) 2–7	3.5 ± 2.35 (3.00) 1–11

Continuous variables are shown as mean ± standard deviation.

Categorical variables are shown as ratio.

TABLE 2: Endometrial thickness in women before and after infusion of G-CSF who had IVF-ET.

Characteristic	All women <i>n</i> = 37	Women who conceived <i>n</i> = 7	Women who did not conceive <i>n</i> = 30
Endometrial thickness before G-CSF-infusion	6.74 ± 1.75 ¹	6.86 ± 1.65 ²	6.71 ± 1.80 ³
Endometrial thickness after G-CSF infusion	8.42 ± 1.73 ¹	8.80 ± 1.14 ²	8.33 ± 1.85 ³
Endometrial thickness (Δ)	1.68 ± 1.05	1.94 ± 0.99 ⁴	1.62 ± 1.07 ⁴

Data are shown as mean ± standard deviation.

P value for two dependent samples (before versus after).

¹*P* < 0.001.

²*P* = 0.0020.

³*P* < 0.0001.

P value for two independent samples (women who conceived versus women who did not conceive).

⁴*P* = 0.8481.

⁵*P* = 0.2444.

⁶*P* = 0.4650.

5. Discussion

It has been demonstrated before that <1% of women have thin endometrium [2, 16]. The thin unresponsive endometrium is still the unresolved clinical problem. There are inconclusive data regarding the diameter of so-called thin endometrium. Some investigators stated that the pregnancy occurs when endometrium reaches more than 7 mm and others that more than 9 mm [2, 3, 15, 16]. However, there are also data in the literature that endometrium 5–8 mm is inadequate [17]. Several methods were proposed, to increase thin endometrium in women undergoing IVF. These therapies included tocopherol, pentoxifylline, low-dose aspirin, sildenafil citrate, and estradiol administration [16, 18, 19].

The embryos of patients with thin endometrium have to be frozen, which leads to the real clinical dilemma for doctors. On the other side, in some investigations, there was no correlation between the IVF outcome and endometrium thickness [20, 21]. In the pilot study of Gleicher et al., the authors showed preliminary clinical report regarding the role of G-CSF on endometrium expansion in women with unresponsive endometrium [13]. In this case report, the data of four patients infused with G-CSF into the uterus were demonstrated. All these patients finally conceived. Two years later, the same group of authors described 21 patients

with inadequate thin endometrium infused with G-CSF. As a result, 19.1% ongoing clinical pregnancy rate was observed. The findings of Gleicher et al. provided evidence that G-CSF could be promising agent in the treatment of women with thin unresponsive endometrium [14].

In our study, we analyzed 37 women who underwent IVF-ET after G-CSF infusion. The clinical pregnancy rate was very similar to Gleicher study [14]—18.9%. We found that the endometrium significantly increased after infusion of G-CSF when we analyzed all the examined women and when we divided them according to the conception success. The increase of endometrium thickness was greater in group of women who conceived but the difference between groups was not statistically significant. These observations were in accordance with the study presented by Gleicher et al. [14]. In that pilot data, the endometrium increased also in all women but more in the subgroup of women who conceived. In contrast to Gleicher et al.'s study, our population was younger (34.6 versus 40.5 years) and had higher AMH concentrations (4.2 versus 1.5 ng/mL). Another difference was the time interval between G-CSF infusion and the first reassessment of the endometrium; in Gleicher et al. [14] study, it was 48 hours in contrast to 72 hours in our study. However, the question what is the appropriate interval between infusion and second reassessment of endometrium is still open. Secondly, we are

still not sure how many times G-CSF should be applied. In Gleicher et al.'s study [14], three patients (14.3%) reached the minimal thickness after the second infusion of G-CSF. In contrast, in our study, we infused G-CSF only once. We also do not know the extent to which the increase of endometrial thickness is the result of G-CSF function or the synergistic effect of added low-dose aspirin and to the protocol. This supplementation was routinely applied in our study. There are opposing results with aspirin in unselected IVF patients on the endometrium thickness and pregnancy rates [22, 23].

Our study is not without limitations. Firstly, we did not have a control group which received placebo. Thus, the changes of endometrial thickness could be observed only before and after infusion and between subgroups of women who conceived or not. Secondly, the subgroup of women who conceived was very small. Thirdly, we applied aspirin and/or sildenafil citrate which also could have a positive effect on endometrial thickness. For example, aspirin attenuates placental apoptosis, and this could be a possible explanation of how aspirin is beneficial, even in the absence of endometrial or oocyte improvement [24]. We can only speculate that the other factors could have impact on endometrial thickness. For example, we did not measure antiphospholipid antibodies [25]. The presence of them could have influenced endometrial thickness during low-aspirin treatment. But we do not believe that this could have essential impact on our results. We think that taking into account all previous failures the G-CSF effect could play the main role in our study. One should note that women who conceived were younger than women who did not but the difference between groups was not statistically significant.

Therefore, the final assessment on how G-CSF affects the expansion of endometrium thickness remains open until prospectively controlled studies would be performed.

To date, no final conclusions have been also drawn regarding which delivery system is better. Despite the aim of our study, we did not compare the assessment of adverse events and did not record any adverse effect during G-CSF infusion. However, it was demonstrated before that the treatment with G-CSF could lead to bone pain, general fatigue, headaches, insomnia, anorexia, nausea, and/or vomiting [26]. Additionally dyspnea, chest pain, hypoxemia, diaphoresis, anaphylaxis, syncope, and flushing were recorded [27]. There is also a question on how to properly counsel the patients who did not conceive and still have thin unresponsive endometrium despite G-CSF infusion. However, we showed some possibilities to the patients.

In summary, we showed that, in women who had thin endometrium in the previous IVF cycles, the infusion of G-CSF increases the endometrial thickness. Additionally, the expanding of endometrial thickness was observed after 72 hours. Because of the limited number of women and no control group, our conclusions are limited. We should also remember that the threshold is different in many other studies; thus, clinical pregnancy was observed even in women with endometrium <4 mm [28]. We think that, despite the obvious limitations, our data are important for doctors and couples seeking fertility assistance. However, further studies are needed in this field.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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