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Infertility

Guest Editors: Mittal Suneeta, Dhaliwal Lakhbir, Sharma Sanjeev, Chauhan Sanjay, B. S. Garg, and Neeta Singh

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Editorial Board

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Editorial

Infertility

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Infertility is acquiring a proportion of global epidemic with the prevalence rate of approximately 8–10% according to World Health Organization. Reproductive health implies individual's right to reproduce and freedom to decide when and how often to have children. The couples have a right to have children and right to access appropriate health care services that will enable them to achieve their goal. Infertility, however, continues to be a worldwide problem, affecting an estimated 60–80 million women and men worldwide, a vast majority of whom live in resource poor countries.

The problem in low-resource countries is compounded because of several factors specially related to unhygienic obstetrics and postabortal practices. Most low-resource countries also have high population growths compared to the developed countries. Increasing population understandably dilutes the health care funding; however infertile couples are denied treatment for their suffering in the name of not increasing the already high population growth, a problem which the infertile couples did not contribute to.

Infertility may not be a threat to physical health but carries with it extremely adverse social and psychological implications for all concerned but particularly in developing countries. Since it is accepted that individuals cannot achieve health in general and reproductive health in particular without the alleviation of infertility there is an ever-increasing need and urgency to develop simple, low-cost, and effective instruments for evaluation, treatment, and prevention of infertility, which can be applied universally.

Infertility thankfully is not considered a "female" only problem; however lack of awareness still ensures that the women bear the brunt of the blame and its associated reactions. Current understanding of the pathophysiology of infertility based on the newer technologies, for example, structural genetics, molecular biology, and imaging techniques has significantly improved the management strategies. This understanding has also made us better aware of the well known pathologies however these need to be justified and applied carefully in low-resource countries making the interventions specific, clinically relevant, and cost-effective.

With idea of focused reading publication of special issues on specific important area is the unique quality of this journal. Infertility is an area that needs specific attention by dedicated physicians working in this specialty. Quality of submitted manuscripts and their review process has been very meticulous. The current issue has looked at the well-known pathologies and reviewed them in the light of the current understanding. This issue highlights the various topics dealing with social, epidemiological, causative, and treatment modalities and recent advances in management of infertility.

Infertility is multifaceted condition with a myriad of causes and treatment. This has been possible due to tremendous research and improvement in the reproductive and genetic technologies. However, it is important not only to have a basic background of facts and fundamental principles, but also revisit and revise our perspectives of treatments for infertility.

Ovulatory dysfunction appears to be on the rise with changes in life style, increase in stress and strain, late child bearing practices, and rising incidence of polycystic ovarian disease. Induction of ovulation remains backbone of infertility treatment, and clomiphene citrate has earned its

fame as ovulation-inducing agents for almost half a century. With now availability of Letrozole (though not approved in many countries yet) ovulation results are as good as clomiphene; in addition women with thin endometrium get good response; the article on its use in polycystic ovary syndrome provides its dosage optimization which so far is not very clear as the dosage of clomiphene is standardized.

Assisted reproduction is one of the fastest growing areas of medicine having expanded far beyond the imaginations of those who pioneered the techniques that led to the birth of Louise Brown. Thirty-odd years after her birth, infertility treatment has improved substantially. Not only has the process of uniting egg and sperm outside the body become a commonly practiced procedure, assisted conception treatments are proving to be more efficient and cost-effective. The availability of cryopreservation technology has extended the scope of infertility treatment and proved to be boon for patients. Assisted hatching, preimplantation genetic diagnosis, ovarian tissue freezing, stem cell technology, gamete donation, embryo donation, and surrogacy, these treatment options are mind-boggling. This requires the healthcare provider to be well equipped with the present scenario. Review article by P. R. Brezina and Y. Zhao has done good justice to ethical, legal, and social issues impacted by modern ART.

Review articles on important chronic benign problem of endometriosis and adenomyosis are very informative. In the era of stem cell therapy the article on endometrial stem cells and reproduction should sensitize the readers to work and do more research in this area since a significant number of women with damaged endometrium would benefit from this mode of therapy especially in developing countries where endometrium is destroyed due to genital tuberculosis in addition to other causes. Studies are required to delineate the mechanisms responsible for successful treatment of Asherman's syndrome, an intractable disease.

Genetic variation and environmental factors contribute susceptibility to spermatogenic impairment in human, the article on sperm DNA integrity assessment reviews use of this new tool in the diagnosis of male infertility. Every patient with tubal factor infertility cannot afford assisted reproduction, and good number of them may benefit from tubal reconstructive surgery, but the postoperative adhesions formation is a big hurdle to success the article on prevention of postoperative adhesions with viscous liquid explores very economical mode of preventive measures and makes an interesting reading. Many more community-based epidemiological studies on the infertility need to be done like the one from Pakistan.

In this special issue the topics have been arranged as epidemiology followed by the causative factors, investigations, infertility management, and its effect/side effects and include both original research and review articles.

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Research Article

Optimal Timing for Oocyte Denudation and Intracytoplasmic Sperm Injection

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Objectives. To analyze the impact of oocyte denudation and microinjection timings on intracytoplasmic sperm injection (ICSI) outcomes. *Study Design*. We included ICSI cycles with the following parameters: rank 1 or 2, female age < 36 years, male factor infertility, long protocol using GnRH agonist and rFSH for ovarian stimulation, and use of freshly ejaculated sperm ($n = 110$). Several ICSI parameters were analyzed according to the time between oocyte retrieval and denudation (T_1) and the time between denudation and ICSI (T_2) using a statistical logistic regression analysis. *Results*. Neither T_1 nor T_2 had a significant influence on the Metaphase II (MII) rate but the fertilisation rate (FR) showed a significant improvement when T_1 was longer (optimal results at $T_1 = 3$ hours) while FR significantly decreased with the increase of T_2. Optimal implantation (IR) and pregnancy (PR) rates were obtained when T_1 was around 2 hours. *Conclusion*. Incubation of oocytes around 2 hours between retrieval and denudation may not increase MII rate but appears to lead to the optimal combination of FR and IR.

1. Introduction

Intracytoplasmic sperm injection (ICSI) is the treatment of choice for couples with severe male infertility. The microinjection technique has been completely standardized but there is no common standard for the precise timings of all the procedures. No more than 7 studies focusing on the influence of ICSI procedure timings on the outcoming results were published [1–8], with discrepancies in the conclusions.

Although it has been shown that incubation of oocytes for 2–6 h prior to IVF (*In Vitro* Fertilization) improves fertilization and pregnancy rates [9–12], there are some conflicting results regarding the timing of ICSI. It has been reported that a preincubation period between oocyte retrieval and injection in ICSI cycles improved the percentage of mature oocytes [5, 6], the fertilization rate [1, 6, 7], and the embryo quality [1, 2]. A long oocyte preincubation (9–11 hours) prior to ICSI is thought to have bad effects on embryo quality [2], probably due to oocyte ageing. However, other studies supported different results as no statistically significant differences in the fertilization [3, 4] or the pregnancy rates [2, 6] were found in preincubated oocytes during ICSI cycles.

In theory, some problems may be associated with the injection time. Oocytes are retrieved prior to ovulation in the procedure of IVF or ICSI. According to some reports [13, 14], preovulatory oocytes are not fully mature, even though a first polar body is present. It is so called the cytoplasmic immaturity. Cytoplasmic maturity is thought to be asynchronous with nuclear maturity in stimulated cycles

[15, 16]. Hence, the fertilizing ability of an oocyte with a mature nucleus is not necessarily at its maximum potential. Therefore preincubation of oocytes prior to IVF or ICSI may induce cytoplasmic maturation that could eventually increase fertilization and also pregnancy rates. Moreover, Balakier and colleagues [14] reported that human oocytes progressively develop the ability for full activation and normal development during the MII arrest stage. The improvement in fertilization rates was obtained when ICSI was carried out 6–8 hours after the first polar body expulsion. For normal fertilization to occur, both nuclear and cytoplasmic maturity is required independently [15].

Whether to carry out the oocyte denudation directly after their retrieval or to keep the surrounding cumulus cells during the preincubation is also not clear in the literature. The only published report focusing on the influence of denudation timing did not show any significant influence on the results [3].

The purpose of this study was to analyze retrospectively the impact of oocyte denudation and microinjection timings on the ICSI outcome in a selected population so as to carry out corrective measures to improve the results.

2. Material and Methods

2.1. Patients. ICSI were performed at Cochin-Saint Vincent Hospital (Paris, France) between January 2004 and February 2007. Only were included in this retrospective study ICSI with the following parameters: attempt rank 1 or 2 of ICSI; female age < 36 years old; male factor infertility (total motile spermatozoa after selection <500 000 or presence of antispermatozoa antibodies IgG > 80% and/or IgA > 80% located on sperm head) long protocol using GnRH and rFSH for ovarian stimulation [17]; oocytes retrieval performed 36.5 ± 1 hour after hCG administration; use for ICSI of freshly ejaculated sperm.

We excluded from this retrospective study cycles where a female infertility could be evidenced as: (i) premature ovarian failure (FSH at Day 3 $\geq$ 10 IU/mL), (ii) <4 oocytes retrieved, (iii) grade III or IV endometriosis according to American Fertility Society classification, or (iv) polycystic ovaries syndrome (ESHRE 2003). We also excluded the cycles during which a long ICSI procedure was mentioned on the laboratory sheet (more than one hour) or ICSI for which more than 15 oocytes were collected to avoid including polycystic ovary or overstimulated patients who might have an underlying oocyte maturation problem. For these reasons and to eliminate a potential bias linked to the sperm origin, ICSI performed with surgically retrieved spermatozoa was excluded from the study.

2.2. Ovarian Stimulation. Ovarian stimulation, ovulation triggering, and oocytes collection were carried out as described elsewhere [17].

2.3. Oocyte Preparation. The cumulus and corona cells were removed using enzymatic digestion after a variable timing following oocyte retrieval then incubated in IVF medium (Medicult, France) at 37°C, 5% CO_2 in air till ICSI was performed. Only morphologically normal-appearing mature oocytes with a visible first polar body by the time of ICSI procedure were microinjected. During all the time of the current study, the ICSI conditions were identical (equipments, media, and culture conditions).

2.4. Semen Preparation. Semen samples were collected the day of oocyte retrieval by masturbation. After liquefaction, semen was prepared by a 90-45 gradient system using Puresperm (JCD, France). Sperm motility and concentration were assessed according to World Health Organization [18] criteria before and after preparation. The timing between the end of sperm preparation and the beginning of ICSI was comprised between 30 minutes and 2 hours.

2.5. ICSI Procedure. Intracytoplasmic sperm injection was performed as described elsewhere [19]. Sperm injections were performed throughout the day, depending of the number of ICSI procedures, and the workload in the laboratory.

2.6. Timing. Different timings were analyzed in this retrospective study (Figure 1). The timing between oocyte retrieval and oocytes denudation was the timing elapsed between the beginning of oocyte retrieval and the beginning of oocyte denudation (T_1). The timing between oocyte denudation and ICSI procedure was the timing elapsed between the beginning of oocyte denudation and of ICSI procedures (T_2). The timing was recorded immediately before each act. The timing of case assignment was random, determined only by the work load on a given day.

2.7. Assessment of Fertilization and Embryo Development. Assessment of fertilization was made 17-18 h after ICSI by checking the number of polar bodies and pronuclei. The fertilization rate was defined as the ratio between the number of diploid zygotes and the number of mature oocytes. After confirmation of fertilization, each normally fertilized oocyte was transferred into a new ISM1 medium 30 μL droplet. Early cleavage was assessed 25 ± 1 hours after ICSI and embryo cleavage was evaluated after a 44 ± 2 hours culture. The cleavage rate was defined as the ratio between the number of embryos and the number of mature oocytes. Embryo grading was performed according to the number and the size of the blastomeres (regular or irregular cleavage), the presence or not of multinuclear blastomeres as well as the percentage of anucleate fragments. Embryos were put into one of 4 categories according to the percentage of anucleate fragments: type A, when there was no anucleate fragmentation, type B when 1–20% of the embryo was fragmented, C when the proportion of fragmentation ranged between 21 and 50% and D when over 50%, of the embryo was fragmented. Embryos were transferred 2 days after oocyte collection.

We considered as "TOP" embryos those seen fertilized at day 1 and were regular 4 to 5 cell embryos at day 2 with less than 20% fragmentation and without any multinuclear blastomeres. The percentage of TOP embryos was defined as

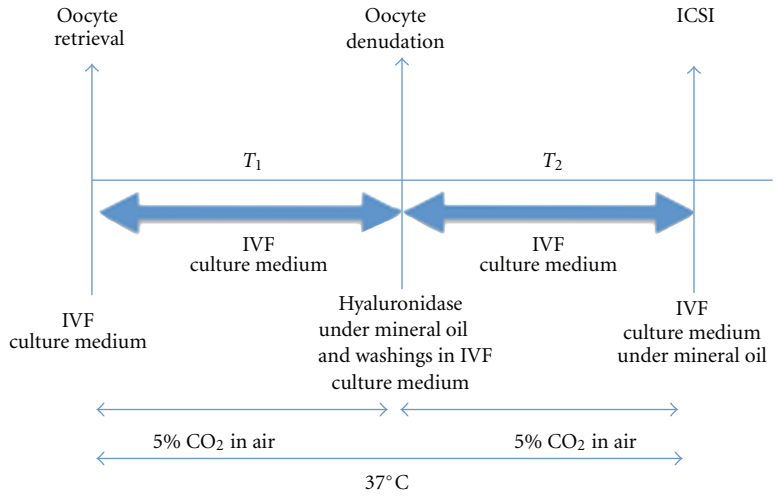

Figure 1: Schematic representation of the two timings T_1 and T_2.T_1: Time elapsed between oocyte retrieval and denudation. T_2: Time elapsed between oocyte denudation and ICSI.

the ratio between the number of top embryos and the total number of embryos.

There were different operators who participated to the gamete and embryo manipulations during the study. There was no difference in the fertilization and implantation rates between the different operators.

2.8. Clinical Pregnancy and Implantation. Pregnancy test using serum hCG assay was performed on Day 12 after em-bryo transfer. Ongoing pregnancy refers to successful progress of pregnancy beyond the 10–12th week of gestation by an ultrasonography confirmation of one or more gestational sacs with heart activity.

The implantation rate (IR) was the ratio between the number of gestational sacs and the number of transferred embryos.

2.9. Statistical Analysis. Quantitative variables are reported as mean-plus or-minus standard deviation, while qualitative variables are reported as frequencies and percentages.

To study the impact of the two timings on various outcomes (fertilization rate, cleavage rate, and embryo quality), logistic regression models were used. Because each patient in our dataset could provide more than one datum, independence of the observations was questionable, and generalized estimated equations were used with exchangeable correlation matrices.

For each outcome, in a first step, we successively constructed (i) a logistic regression model to study the impact of the timing between oocytes retrieval and denudation and (ii) another model to study the impact of timing between oocytes denudation and ICSI injection. To account for potential nonlinear effects of both timings, second and third order polynomial terms were entered together with linear terms. Effects were considered significant if the corresponding P values were less than 0.05. Then, all significant terms obtained in the first step along with their interactions were included in another logistic regression model to study the independent impact of both timings. Again, effects were considered significant if the corresponding P values were less than 0.05.

3. Results

3.1. Patients and ICSI Cycles. A total of 110 of the 1691 (6.5%) ICSI cycles performed during this period met the criteria of inclusion. The results of ICSI from 110 treatment cycles are shown on Table 1. Mean sperm parameters after selection were as follows: $10.1.10^6 \pm 1.4$ sperm concentration, $40.6 \pm 2.9\%$ progressive motility. They were not significantly different between each timing investigated (data not shown). A total of 1230 oocytes were obtained (11 ± 3.0 oocytes per retrieval), 903 oocytes (73.4%) were mature just before ICSI. The overall fertilization rate (FR) of the injected oocytes was 65.8% and 593 embryos (65.7% of cleavage rate) were obtained, with 25.8% of them considered as TOP embryos. Forty-six of the injected oocytes were not mature at denudation and so matured *in vitro* between denudation and ICSI. They represented 5.0% of the injected oocytes and their proportion did not significantly varied according to the different timings. The FR of the *in vitro* maturated oocytes (22/46 47.8%) was significantly lower comparing to the ones already mature at denudation (572/857 66.7%; $P = 0.008$, χ^2 test).

A total of 107 embryo transfers were performed with a mean number of 1.7 ± 0.3 embryos transferred (196 transferred embryos). These transfers led to 56 fetal sacs, 47 ongoing pregnancies, 37 live births, 7 miscarriages, 1 neonatal death following eclampsia, 1 unknown outcome, and 1 medical abortion for oligohydramnion.

3.2. Effect on the ICSI Parameters. The statistical analysis of the data showed no statistical differences according to the time of denudation and microinjection on the MII

Table 1: ICSI cycle characteristics and outcomes.

Number of cycles	110
Number of oocytes (mean [range])	1230 (11.2 [5–27])
Number of MII oocytes at the injection (%) (mean [range])	903 (73.4%) (8.2 [4–15])
2PN fertilized oocytes (%) (mean [range])	594 (65.8%) (5.4 [4–15])
Number of degenerated MII after the injection (%) (mean [range])	43 (4.8%) (0.4 [0–4])
Number of obtained embryos (%) (mean [range])	593 (65.7%) (5.4 [0–14])
Number of TOP embryos (%) (mean [range])	153 (25.8%) (1.4 [0–7])
Number of transferred embryos (mean [range])	196 (1.8 [0–2])
Number of gestational sacs	56
Implantation rate	28.5%
Number of clinical pregnancies (per cycle %)	47 (42.7%)
Clinical pregnancy per embryo transfer	43.9%
Live birth (per cycle %)	37 (34.6%)

percentage (data not shown). The fertilization probability $P(f)$ significantly increased with the increase of T_1 ($P < 0.0001$; Figure 2). The time T_1 had consequently a significant effect on the fertilization but this effect was not linear. The maximum of probability of fertilization was at an interval of 3 hours between oocytes retrieval and denudation. On the other hand, the relation binding the time between oocyte denudation and microinjection (T_2), $P(f)$ was linear and statistically significant (Figure 2). But on the contrary the fertilization probability decreased with the increase of the time lasting between oocyte denudation and injection so the shorter T_2 was, the better fertilization results that we had. Consequently, the combined effects of both periods T_1 and T_2 were influencing significantly the fertilization outcome. So, according to the fertilization rate results, the optimal timings are to perform the oocyte denudation within 3 hours after oocyte retrieval and to perform sperm microinjection without any delay after oocyte denudation.

In terms of embryo quality, there was not any statistical significant influence of both procedure timings on the percentages of TOP quality embryos (data not shown).

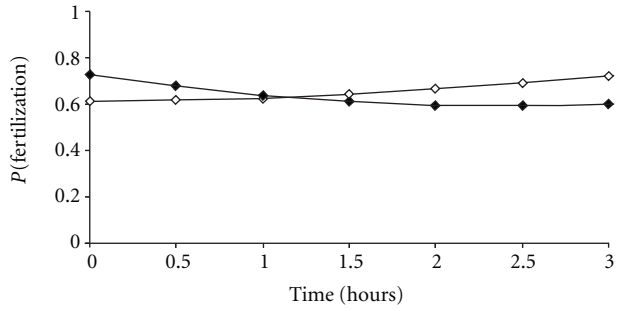

Figure 2: Influence of the timing between oocyte retrieval and denudation (white box) and the timing between oocyte denudation and ICSI injection (black box) on the fertilization rate. The relation between P(fertilization) ($P(f)$, the probability of the oocytes to be fertilized) and the time between oocyte retrieval and denudation (T_1) was statistically significant with a P value <0.0001 related to $(T_1)^2$. $\log(P(f))/(1 - P(f)) = 0.46 + 0.06\ (T_1)^2$. The relation between P(fertilization) ($P(f)$, the probability of the oocytes to be fertilized) and the time between denudation and microinjection (T_2) was statistically significant and linear with a 0.0083 P value related to T_2, following the equation $\log(P(f))/(1-P(f)) = 0.97 - 0.50(T_2) + 0.11\ (T_2)^2$and with a 0.005 P value related to $(T_2)^2$.

3.3. Effect on the ICSI Outcomes. In terms of pregnancy results, both T_1 and T_2 did not have any statistically significant influence on pregnancy outcomes (data not shown for T_2) but when representing the probability of pregnancy according to the time elapsing between oocyte retrieval and denudation (Figure 3(a)), it appeared that we had optimal results when denudation is achieved around 2 hours after oocyte retrieval. When considering the probability of delivery according to the same timings, it followed the same tendency. The same effect was observed whether one or two embryos were transferred with a higher pregnancy rates reached at 2 hours.

The time elapsed between oocyte retrieval and denudation had a significant impact on implantation rates with optimal results when oocytes were denudated around 1.5–2 hours after retrieval (Figure 3(b)). With regards to the time elapsed between denudation and microinjection, there was no statistically significant impact on the implantation results (data not shown).

4. Discussion

In the literature, among six studies interested in ICSI procedure timings [1, 2, 4–7], none analyzed the impact of both oocyte denudation and microinjection timings on ICSI outcomes. Most of them assumed that oocytes should be incubated *in vitro* surrounded by the corona and cumulus cells [1, 2, 6, 7]. Moreover, they stated that the denudation was achieved directly prior to ICSI but there is no evidence showing whether it is preferable to keep the cells surrounding the oocytes during preincubation and which timing it should be adopted. The originality of our study design was to separate the timing between the procedures into two successive

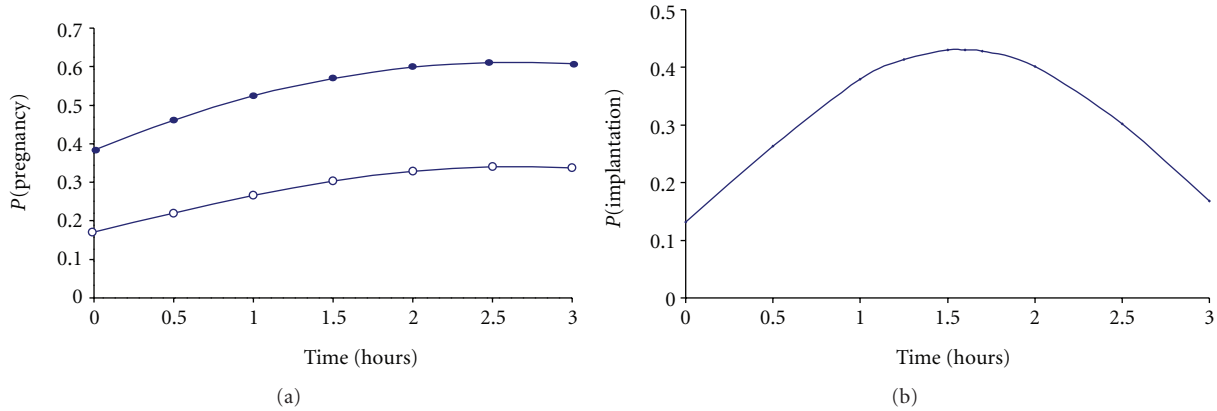

FIGURE 3: Influence of the timing between oocyte retrieval and denudation on the pregnancy rate (a) and the implantation rate (b). (a) The relation between the probability of pregnancy P(Pregn) and the time between oocyte retrieval and denudation (T_1) follows the equation $\log(P(\text{Pregn}))/(1 - P(\text{Pregn})) = -2.69 + 0.70(T_1) - 0.13(T_1)^2 + 1.11$ but was not statistically significant (P values: (T_1): 0.1364, $(T_1)^2$: 0.1232). Two curves are represented depending on how many embryos are transferred $R = 1$ (white circle) if 1 embryo is transferred and R = 2 (black circle) if 2 embryos are transferred. (b) The relation between the probability of implantation P(i) and the time between oocyte retrieval and denudation (T_1) was statistically significant following the equation $\log(P(\text{i}))/(1 - P(\text{i})) = -1.89 + 2.04(T_1) - 0.65(T_1)^2$ (P values: (T_1): 0.03 and $(T_1)^2$ 0.03). The best implantation rate was observed for a time between oocyte retrieval and denudation around 1.5 hours.

periods: T_1 and T_2, to study distinctly the optimal timing of each procedure to improve ICSI results. Accounting for all the results, the denudation should be achieved at least 2 hours and up to 3 hours after oocyte retrieval for optimal fertilization and implantation results. ICSI should be achieved as soon as the denudation is completed.

We confirmed previous report [3], as we did not find any influence of the time between oocyte retrieval and denudation nor between denudation and ICSI on the percentage of meiotically mature oocytes. Other studies found that the preincubation of oocytes *in vitro* prior to denudation and/or injection is beneficial as we get more mature (MII) oocytes [5, 6]. Ho et al. [5] even stated that it was the only improvement that he got from pre-incubating oocytes prior to ICSI.

Even if no differences were observed in the percentage of meiotically mature oocytes in our study, significant differences were observed in fertilization rates. A longer incubation period prior to denudation (up to 3 hours) gave better fertilization rates. This could mean that a delay of denudation may not necessarily increase the number of meiotically mature eggs but it allows the MII oocytes to complete cytoplasmic maturation. Nuclear maturity can easily be assessed before ICSI as it is evidenced by the expulsion of the first polar body but the cytoplasmic maturity process is not very well known, it is thought to involve maternal mRNA and proteins [4]. In natural cycles, nuclear and cytoplasmic maturity is highly coordinated whereas in stimulated cycles, the two phenomena appear to be asynchronous [15, 16]. Hence, some oocytes retrieved from stimulated cycles could be cytoplasmically immature despite reaching the MII stage. This could be one explanation for the fact that the timing between oocyte retrieval and denudation influences significantly the implantation rate (with best results when denudation is achieved around 1.5–2 hours after oocyte retrieval) even though the embryo quality was not affected by the timing of the different procedures. The surrounding cells might also secrete paracrine substances and growth factors or express adhesion molecules on their surface membranes that might play a role in the nuclear and/or oocyte maturation. For example, the ovarian brain-derived neurotrophic factor secreted by granulosa and cumulus cells is essential for nuclear and cytoplasmic oocyte development [20].

Conflicting results concerning the preincubation of oocytes prior to ICSI were found in the literature. Some supported it [1, 2, 5, 6], others did not find evidence showing any advantages [3, 4]. And, even among those studies which support the incubation of oocytes prior to ICSI, it does not always concern the same parameters, upregulating either the fertilization rate only [1, 7], the embryo quality only [2], or both fertilization and pregnancy rates [6]; (our study). Furthermore, there is no common agreement on how many hours the oocytes should be incubated. We intentionally analyzed data of ICSI performed on a selected population to avoid possible factors that could bias the results. Among them, a fixed timing after hCG administration was determined (36.5 h ± 1 post hCG). In fact, the majority of the oocyte retrievals were performed between 36.0 and 37.0 hours, that is, 36.5 h ± 0.5 post HCG as only 13 (11.8%) was out of range. But the same conclusion was obtained after reanalysing the data without these 13 attempts. The time between oocyte retrieval and ICSI should not also exceed a certain sensible limit. In some studies, oocytes were injected up to 11 hours [1, 2] after retrieval, which seemed to have a bad influence on the embryo quality as significantly less good quality embryos were obtained from the oocytes injected 9–11 hours after retrieval [2]. It has been shown that ageing oocytes are much more sensitive to parthenogenetic activation [21] or that a longer preincubation period could affect oocyte quality [22]. The capacity of the cytoplasm of MII oocytes to decondensate sperm DNA, resume meiosis, and promote the evolution of male pronucleus appears to decline progressively 24 hours after oocyte retrieval [23].

Van de Velde claimed that ICSI should not be done more than 12 hours after retrieval because *in vitro* ageing seems to result in spindle instability and subsequent loss or scattering of chromosomes in the oocyte [24–26].

5. Conclusion

Our results suggest that the preincubation between oocyte collection and denudation up to 3 hours after retrieval in ICSI may not increase the percentage of mature oocytes but improves the fertilization and implantation rates even though the embryo quality is not influenced. On the other hand, the sperm injection should be achieved without any delay after oocyte denudation to keep good fertilization results.

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Research Article

Perceptions and Experiences of Women in Karachi, Pakistan Regarding Secondary Infertility: Results from a Community-Based Qualitative Study

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Background. The prevalence of infertility in Pakistan is 22% with primary infertility at 4% and secondary infertility at 18%. This study explored perceptions and experiences of women in Karachi, Pakistan regarding the causes, treatment-seeking behavior for and consequences of secondary infertility. *Methods*. Focus group discussions and in-depth interviews with married women explored their perceptions and experiences for issues related to secondary infertility. *Results*. The knowledge of women about the causes and scientific treatment options for infertility was limited resulting in inclination for traditional unsafe health care. Infertility was stated to result in marital instability, stigmatization and abuse specially for women with no live child. *Conclusions*. Since infertility can have a serious effect on both the psychological well-being and the social status of women in Pakistan, effective interventions are the need of the day. There is a dire need for health education and counseling to be integrated into infertility management plans.

1. Introduction

Infertility is defined if a couple is unable to achieve pregnancy for at least one year of unprotected intercourse without using any contraceptives [1]. Globally, 10–15% of couples of reproductive age are infertile and prevalence varies from country to country. However, the trend of secondary infertility outnumbering the primary infertility is similar across all the developing countries [1, 2] with postabortal, puerperal [3, 4], and reproductive tract infections (RTIs) as the major causes. RTIs are either sexually transmitted [5] or result from infections introduced through medical procedures (during IUCD insertion, inducing an abortion or labor) [6]. Infertility has been viewed to be associated with increased psychological distress for the couples [7] with greater social pressures for women than men [8]. This is more marked for women with secondary infertility who either are unable to conceive again or face poor pregnancy outcomes such as abortions or stillbirths. Studies have shown that in these situations women not only are harassed by the family members but, face various forms of marital instabilities too [9, 10]. In Pakistan, the overall prevalence of infertility is 22% with primary infertility at 5% and secondary infertility at 18% [11]. There is a dearth of information about causes of infertility and health seeking behavior of infertile couples in the country.

A study was conducted to explore the perceptions and experiences of women regarding causes of, treatment-seeking behavior for, and consequences of secondary infertility. This paper describes the findings of the qualitative component.

2. Methods

2.1. Study Design. The qualitative research methods were used for better understanding of the contextual issues surrounding women's perceptions and experiences for causes and consequences of and health seeking behavior for secondary infertility. Focus Group Discussions (FGDs) and In-Depth Interviews (IDIs) were conducted to collect the information which gave a flavor of mixed methods.

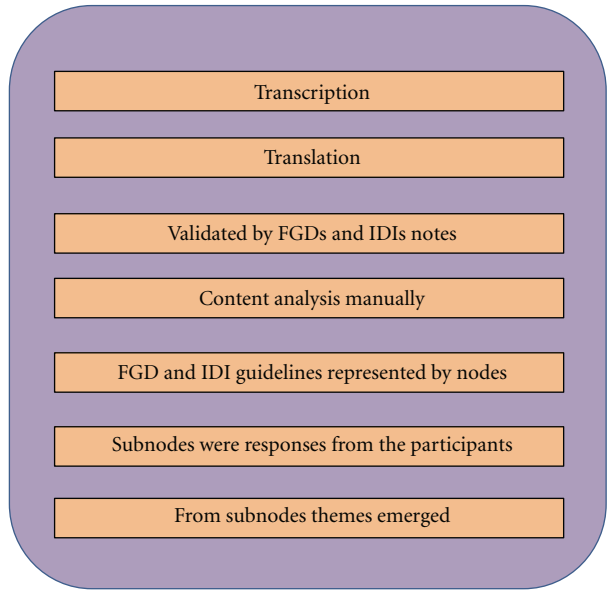

Figure 1: Analytical process of FGDs and IDIs.

2.2. Study Setting. The community-based study was conducted in urban areas of Karachi, Pakistan. The communities chosen were in the vicinity of selected infertility clinics. The data was collected from April to June 2006.

2.3. Study Participants. Purposive sampling methodology was used [12] to select currently married women aged 15–35 years for the study. FGDs were conducted with women having at least two live children to delve into the perceptions of fertile women about infertility. IDIs explored detailed experiences of women facing secondary infertility. The two methods demarcated the differences and similarities in perceptions and experiences. The local community-based health workers helped in identifying participants according to the selection criteria.

2.4. Process of Data Collection. Separate guidelines for FGDs and IDIs were developed in English, were translated into local language, and were pretested. The guidelines for FGDS and IDIs explored the participants' perspectives and experiences for causes (physical causes, menstrual irregularity, infections, etc.) and consequences of and health-seeking behavior for secondary infertility, respectively.

Ten FGDs and twenty IDIs were conducted. Each FGD had 6–10 participants. PI facilitated the discussions and interviews which were audio-taped. Notes were taken by note keepers. The data collection continued till the point of saturation when no new data was emerging [13].

2.5. Ethical Consideration. Before starting the data collection, ethical approval was obtained from the Ethical Review Committee of The Aga Khan University, Karachi. Written informed consent was obtained from all participants. The consent form was read for illiterate participants before having their thumb impressions. The consent form was signed by the PI, facilitator of the discussion/interviews, and by a witness.

2.6. Data Analysis. All the written and recorded materials were transcribed and translated into English. The data analysis started simultaneously with the data collection and was an iterative and continuous process. The analysis followed the analytic hierarchy, from data management to descriptive and explanatory account, as discussed by Spencer [12, 14].

Content analysis was done manually. The core FGD and interview guidelines were represented by nodes. From these responses (subnodes) and relevant themes were extracted. (Figure 1).

The identified themes were pooled together and integrated to generate concepts for study findings. The research team discussed and identified patterns, commonalities, and differences. During the second stage, the range of perceptions, views, and experiences within an identified theme were analyzed. Finally, links between experiences, behavior, perspectives, and characteristics of the participants were identified. To further ensure trustworthiness, triangulation of methods was undertaken by validating the findings from FGDs, IDIs, observation of group dynamics, and literature review. Results were discussed with qualitative researchers outside the research team.

3. Results

3.1. Sociodemographic Characteristics. Ten FGDs had total of 84 participants while IDIs were conducted with 20 women. (Table 1). The mean age of the participants of FGDs was 26.9 + 5.0 years and that of respondents of IDIs was 26.5 + 4.0 years.

Table 1: Sociodemographic characteristics of women participants of FGDs and IDIs.

	Participants of FGDs ($n = 84$)		Respondents of IDs ($n = 20$)	
	n	%	n	%
Current age (years)				
<20	8	9.5	0	0.0
21–25	24	28.5	8	40.0
26–30	28	33.3	8	40.0
31–35	24	28.5	4	20.0
Ethnicity				
Urdu speaking	28	33.3	10	50.0
Pathan	18	21.4	4	20.0
Punjabi	18	21.4	2	10.0
Sindhi	20	23.8	4	20.0
Respondent's educational qualification				
Illiterate	34	40.4	8	40.0
Primary	30	35.7	8	40.0
Secondary and +	20	23.8	4	20.0
Husband's education qualification				
Illiterate	20	23.8	2	10.0
Primary	36	42.8	6	30.0
Secondary and +	28	33.3	12	60.0
Respondent's occupational status				
Housewives	58	69.0	16	80.0
Employed	26	31.0	4	20.0
Husband's occupational status				
unemployed	6	7.2	0	0.0
Employed	78	92.8	20	100.0

The participants belonged to various ethnicities. Majority of the women were housewives and were either illiterate or had completed their primary education.

Important themes, which were highlighted through these discussions and interviews, are as follows (Table 2).

3.2. Causes of Infertility. The study participants gave a series of explanations for the causes of infertility categorized as follows.

3.2.1. Magic and Spiritual Effects. The FGDs participants perceived magic and spiritual effects (local terminologies: *asaib and saya*) as main causes of secondary infertility among women. An evil spirit was held responsible which affects the women only and cannot influence a man. A participant mentioned that

> "evil spirit lives in woman's body only as it affects the menstrual blood. Since men do not menstruate, it cannot live in their body"

(from FGD 5, woman aged 29, illiterate, mother of 3 children).

Some participants mentioned that the main cause of secondary infertility is a special action (*bandish*), done by spiritual healers, that shackles women's chances to conceive.

The IDI respondents had similar views. While 12 women held evil spirits responsible, the remaining 8 reported menstrual irregularities, swelling/tumor, and wrong position of uterus as the cause. Interestingly, they believed that the physical causes were due to "*bandish*". Those who had faced abortions or stillbirths believed that "*bandish*" "killed" their live pregnancies.

Regarding any anti-*bandish* remedies, the women believed that only special spiritual healers can offer such remedies against huge monetary compensation. It was interesting to note that both literate and illiterate women had similar beliefs.

Table 2: Common themes from analysis of FGDs and IDIs.

(1) Causes of secondary infertility
  (i) Magic and spiritual effects
  (ii) Unhygienic practices during menstruation, intrapartum, and postpartum period
  (iii) Contraceptive use
  (iv) Termination of pregnancy
  (v) Sexually transmitted infections
  (vi) Causes in Men
(2) Treatment seeking behavior
  (i) Time period before seeking care
  (ii)Selection of health care provider
(3) Consequences
  (i) Social consequences
  (ii)Psychological consequences
  (iii) Economic consequences
(4) Adoption as a coping mechanism

3.2.2. Unhygienic Practices during Menstruation, Intrapartum, and Postpartum Period. Regarding unhygienic practices during childbirth, menstruation, and postpartum period and resulting infections as causes of infertility, almost all FGDs participants negated and declared these as "dirty time periods and practices". No one was aware that unhygienic practices during these phases could end up in infections and infertility.

> "How can one have safe practices during dirty periods?"

A participant (FGD 2, aged 31 years, mother of four children, had primary education) asked.

IDI respondents were explored for their practices during menstruation, last childbirth, and puerperium. 18 respondents had home deliveries by untrained birth attendants and were not aware of hygienic care by them. 7 IDI respondents used intra-vaginal home-made medicines during last puerperium.

"These are good medicines for stopping bleeding and relieving pains", a woman reported (IDI 3, illiterate, aged 25 and mother of 1 child).

Regarding menstrual practices, 17 IDI respondents reported the use of clothes, washed and dried in dark areas of the homes to hide these from others.

3.2.3. Use of Modern Contraceptives. The FGDs participants declared that use of any contraceptive could result in infertility. Two participants mentioned infection following IUCD insertion can cause infertility. None of the IDI respondents reported using IUCD.

3.2.4. Attempts for Terminating Pregnancy. The FGDs participants proclaimed an attempt for terminating pregnancy as a cause of secondary infertility and opined

> "Termination of pregnancy is a sin, resulting infertility is the price for that".

(FGD 3, woman aged 30 years, had 3 children and completed 9th class).

None of the IDI respondents reported any attempts for pregnancy termination. However, a woman mentioned "I consulted a TBA for a method for abortion but did not use any. My pregnancy aborted spontaneously. This is the punishment from God that I did not conceive again" (IDI 7, illiterate woman aged 35).

3.2.5. Sexually Transmitted Infections (STIs). Majority of the FGD participants did not know STIs as a cause of secondary infertility. A few participants thought that women with vaginal discharge and backache might face infertility as these cause weakness of the uterus.

"A weak uterus cannot retain a fetus and results in infertility", a woman explained (FGD 7 woman aged 34 years, mother of 2 children, completed primary education). Women were not aware of any STIs among men.

5 IDI respondents reported history of foul smelling vaginal discharge and/or lower abdominal pain. However, none knew about any symptoms of STIs for their husbands.

3.2.6. Causes in Men. All women from FGDs agreed that men cannot be responsible for secondary infertility as he proves to be "fine" by impregnating his wife. "If a man has erection power, he has power of reproducing. If woman has a stillbirth or does not conceive again then man cannot be blamed", a FGD participant viewed (FGD 9, woman aged 29, illiterate, mother of 2 children).

IDIs respondents shared similar views. A respondent reported,

> "My husband said that men cannot be infertile if woman had conceived once so there is no need for my investigation"

(IDI 12, woman aged 34 years, had completed primary education).

3.3. Treatment Seeking Behavior. The perceptions of FGDs participants and experiences of IDI respondents were similar that a woman with a live child waits for 2-3 years before seeking treatment but seeks treatment within 6 months if previous pregnancies ended in abortions/stillbirths. An IDI respondent sought treatment 2 months after her abortion.

3.3.1. Selection of Health Care Providers. The FGD participants perceived that infertile women always consult physicians and gynecologists.

> "This is a modern time and infertile women consult doctors only. In villages, women still opt for traditional healers,"

a woman opined (FGD 8, woman aged 34 years, mother of 3, and completed primary education).

All women agreed that if infertility is due to "*bandish*", then woman should consult a spiritual healer.

The experiences of IDI respondents were different and physicians were not their first choice. Women consulted traditional healers (Traditional Birth Attendants (TBAs), *hakeems* (*a type of traditional healer*), *and homeopathics*) and spiritual/religious before a physician.

The infertile women declared TBAs to be the favorite providers for infertility as they do not advise for any investigations and the prescribed traditional medicines are inexpensive and free of side effects. They mentioned that treatment by physicians/gynecologist is a long process with a series of investigations and treatment. An infertile woman complained

> "Husbands do not agree for investigations and physicians do not treat the wife alone so women stop consulting gynecologists and go to TBAs."

Infertile women sought treatment from spiritual healers too. Two women consulted religious persons (*Pir Sahib)* to get a sacred locket (*taweez*) for tying around lower abdomen,

that is, the place of uterus. Three women visited the tomb of some saint every Thursday night for consecutive 7 weeks. Other women reported the use of sacred water with herbs for drinking and/or vaginal douching.

3.4. Consequences of Secondary Infertility. The consequences of secondary infertility are discussed under the following headings.

3.4.1. Social Consequences. The FGD participants agreed that woman has to bear the brunt of infertility and infertile women get threats for divorces, ejection from homes, and husbands' remarrying. All IDI respondents mentioned that they were being blamed for infertility and were threatened for husbands' second marriages. However, the negative attitude was less marked for women with a live son.

A woman with three stillbirths complained that at the occasions of marriages, she is kept away from the bride as she could transmit the "bad omen" to the bride.

Almost all infertile women with one live child, though thanked God for that blessing, felt that in Pakistani society having one child is equally a stressful. They said that their only child questions for not having any siblings. Two infertile women specifically talked about the high hopes of their families from the only child and feared immense pressure for him/her.

3.4.2. Psychological Consequences. The FGDs participants mentioned depression, unhappiness, and dejection being prevalent among infertile women. Since men are not infertile, the participants assumed that they do not suffer as "he has always a chance to marry again", a participant (FGD 1, woman aged 29, mother of 5 children) thought.

The infertile women agreed to be desperate and anxious for childbearing and lack of support from families/husbands and society's negative attitude instigates the thoughts for committing suicide. An infertile woman (IDI 6 aged 32 years) reported such attempt once.

3.4.3. Economic Consequences. The perceptions and experiences for economical consequences were different. The FGD participants thought that one can pay "infinitely" to have a child. The infertile women found investigations and treatment for infertility to be expensive, and sometimes, unaffordable. One reason for opting spiritual/traditional remedies was their low cost.

3.5. Adoption as a Coping Mechanism. The FGD participants perceived that women with secondary infertility do not opt for adoption as she has been pregnant before and is optimistic to conceive again. Overall, participants felt that an adopted child is usually not accepted in a community. Majority of the IDIs respondents favored adoption but reported resistance by husbands and in-laws. The infertile women declared that their in-laws would support husband's remarrying over an adoption.

4. Discussion

The results of the qualitative component of the study have brought up various important conclusions. However, these could not be generalized as the respondents were selected purposively. Generally, in exploratory study associations can only be suggested and not demonstrated.

Infertility, whether primary or secondary, is labeled as a "disease" of woman and most of the time men are never investigated and treated. This is similar to the findings of other studies [15, 16].

Generally there was a lack of awareness regarding the causes of secondary infertility. Women were not aware of and rather negated any role of unhygienic practices ending up in puerperal and postabortal infections, PID, and infertility. Results from studies conducted in Africa, Bangladesh, and India have revealed unsafe practices as risk factors for infections, PID, and infertility [17–19]. This is particularly alarming in Pakistan where TBAs conduct majority of the deliveries [20] and unsafe abortions are common [21]. Such evidences are indicators for unhygienic practices.

Our study revealed that women's beliefs about superstitious causes of infertility influenced their health seeking behavior too. The studies conducted in Bangladesh and Nigeria have shown similar findings where the cause of infertility is assumed to be due to effects of bad evils resulting in seeking care from spiritual healers [22–24]. However, none of the studies have revealed if some strategies have ever targeted towards such beliefs to modify the behaviors.

The study has shown that though infertile women consult any category of providers, traditional untrained providers were a preferred choice for them. Other studies from Pakistan, India, and Nigeria have shown that traditional healers form quite a popular set of service providers for infertile women [16, 25]. Since the efficacy of both traditional and spiritual treatment has not been proven yet, approaching these healers causes delay in receiving the cause-based scientific management or could possibly be associated with worsening of infertility.

Regarding its consequences, the study has clearly shown that secondary infertility not only socially stigmatizes the women but also challenges their marital stability. Biological motherhood has always being viewed by society as an obligatory part of such relationships [26, 27].

The study has explored adoption as a coping mechanism for infertility. In the developed world, adoption has always been a choice for infertile couples [28]. Almost half of the women with secondary infertility in our study were willing to but could not adopt a child due to the resistance from the husband's family. In Pakistan, child adoption needs to be fully explored for infertile couples as there could be resistance in the families and communities due to perceived religious reasons [29] and fear of disloyalty by the child and claim by the biological parents.

5. Policy Implications

In the light of findings of the study, various policy and advocacy initiatives are required to address the issue of

secondary infertility. There is a need for health education of the community for the correct knowledge of causes of secondary infertility, appropriate time for health seeking, and role of hygienic practices during menstruation, intrapartum, and postpartum periods so that problem could be prevented all together. Infertile couples spend substantial time and money for obtaining healthcare. Seeking traditional and spiritual remedies may result in certain complications due to their nonscientific use and causes delay in getting modern health care. Reproductive health programs should address these issues by training the health professionals at primary health care level. Additionally, it is important for health professionals to realize the stress and anxiety of infertile couples caused by persistent infertility and by undergoing complex investigations and treatment. The attending physician should be trained to give appropriate counseling and support to such couples. In Pakistan, further research in context of infertility in general and secondary infertility in particular would be an important step towards highlighting the extent of the problem and consequences. This would not only help in documenting the preventable causes and so help in precluding and decreasing the prevalence of the problem but would also address the issues related to psychological well-being and the social status of women in Pakistan.

Conflict of Interests

The authors declare that there is no conflict of interests as defined by the guidelines of the International Committee of Medical Journal Editors (ICMJE; http://www.icmje.org/).

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Research Article

Ovarian Volume Correlates Strongly with the Number of Nongrowing Follicles in the Human Ovary

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A reliable indirect measure of ovarian reserve for the individual woman remains a challenge for reproductive specialists. Using descriptive statistics from a large-scale study of ovarian volumes, we have developed a normative model for healthy females for ages 25 through 85. For average values, this model has a strong and positive correlation ($r = 0.89$) with our recent model of nongrowing follicles (NGFs) in the human ovary for ages 25 through 51. When both models are log-adjusted, the correlation increases to $r = 0.99$, over the full range of ovarian volume. Furthermore we can deduce that an ovary of 3 cm^3 volume (or less) contains approximately 1000 NGF (or fewer). These strong correlations indicate that ovarian volume is a useful factor in the indirect estimation of human ovarian reserve for the individual woman.

1. Introduction

The human ovary contains a fixed pool of nongrowing follicles (NGFs) maximal at 18–22 weeks gestation that declines towards menopause when fewer than one thousand NGFs are present [1]. The age at menopause in western populations is 50-51 years on average [2, 3]. Recent socioeconomic changes have resulted in an increasing number of women delaying childbirth until later in life, when their fertility is significantly compromised compared to younger women. This has created significant pressure on fertility services and an increased demand for assisted conception treatments (ACTs).

The assessment of ovarian reserve for the individual woman remains problematic. Direct estimation of the number of NGFs remaining in an individual ovary is currently impossible *in vivo*. A number of physical and humoral factors have been investigated in isolation and in combination. The measurement of follicular stimulating hormone (FSH) in the early follicular phase of the menstrual cycle is an accurate indicator of ovarian function but is not a good predictor of time remaining to menopause [4]. More recently, anti-Mullerian hormone (AMH) has shown promise as a measure of ovarian reserve [5, 6], and transvaginal ultrasound estimation of antral follicle counts (AFCs) is both a useful indicator of ovarian function and reserve [7]. The role of ovarian volume in the assessment of ovarian reserve remains uncertain, with some studies suggesting that a reduced volume is a good predictor of poor outcome for assisted conception [8, 9].

Ovarian volumes increase exponentially from birth to pubertal ages and are believed to be at a maximum shortly after puberty [10, 11]. Many studies have been published using ovarian volumes taken from women either attending infertility clinics or having polycystic ovarian syndrome; Lass and Brinsden have published a detailed survey of these [12]. There have been few studies on the ovarian volumes of groups of women who approximate the healthy population. Most of these were small-scale studies, with exemplars taking measurements from 38 [13] and 377 [14] subjects. One study dominates in terms of size and scope. Pavlik et al. recorded 58,673 volumes from 13,963 subjects taking part in an ovarian cancer-screening project [15]. Each ovary was measured in three dimensions via transvaginal ultrasound. Volumes were calculated using the formula for a prolate ellipsoid: $L \times H \times W \times 0.523$. We consider this study to

Table 1: Curve-fitting results for 10 constructed datasets.

Dataset	Highest-ranked sigmoidal model				Highest-ranked model	
	Height	Centre	Width	r^2	Parameters	r^2
1	1.07	47.1	−13.7	0.667	13	0.678
2	1.07	47.0	−13.6	0.671	12	0.681
3	1.08	46.9	−13.7	0.669	12	0.679
4	1.07	47.0	−13.6	0.669	13	0.680
5	1.07	47.1	−13.6	0.672	13	0.682
6	1.08	46.9	−13.7	0.669	12	0.683
7	1.08	46.8	−13.6	0.669	12	0.680
8	1.07	47.0	−13.7	0.672	12	0.682
9	1.07	46.9	−13.7	0.668	13	0.678
10	1.07	47.0	−13.7	0.668	12	0.678

be the most comprehensive and therefore use it as our sole reference for ovarian volumes.

The aim of this paper is to correlate ovarian volumes as measured by transvaginal ultrasound with our recent description of the decline of the NGF population in the human ovary. Lass and Brinsden have shown that women with small ovaries (less than 3 mL) had a more than 50% risk of abandonment of IVF cycle before NGF retrieval, and that those who did not abandon required more aggressive stimulation than normal [12]. A close, positive correlation between volumes and NGF counts would provide further evidence that women with small ovaries (irrespective of age) are less likely to respond well to ACT.

2. Materials and Methods

Summary statistics were extracted from the Pavlik et al. study [15]: for each year of age from 25 through 85 we obtained the mean ovarian volume, the upper standard deviation, and the number of observations. The total number of observations was 58,255. We derived the log-normal mean and standard deviation for each year of age using standard equations. Parametric bootstrapping is a standard statistical technique for simulating datapoints from a known distribution [16]. The *R* statistical package (The *R* Foundation for Statistical Computing, Vienna, Austria) has a parametric bootstrapping function that returns a fixed number of random deviates from given log-normal means and standard deviations. We used this function to create 10 datasets each having the same descriptive statistics as the Pavlik et al. study (so that each dataset reproduces their published results).

For ages 25–85, inspection of the Pavlik et al. plots shows that ovarian volumes appear to progress from high values declining to a minimum with increasing age. We therefore fitted seven sigmoidal (or "S-shaped") models to the datasets using TableCurve2D (Systat Software Inc., Chicago, IL, USA) and ranked the returned models by the r^2 coefficient of determination. We also fitted 3,164 arbitrary and biologically nonplausible models to the same datasets in order to determine the maximum r^2 obtainable for that data.

We produced a normative model of ovarian volumes from the highest-ranked sigmoidal models and calculated the correlation coefficient, r, for mean ovarian volume against mean NGF population as given by the Wallace-Kelsey model [1] for ages 25 through 51 (since the highest age used in the derivation of the NGF model was 51 years, and the lowest age in the Pavlik et al. study was 25 years). For this correlation, neither quantity was log-adjusted. Variability increases with both ovarian volume and NGF population; hence both quantities are log-normally distributed. Therefore, in order to test correlation between predictive intervals as well as mean values, we log-adjusted both models and calculated correlation coefficients for mean values and upper and lower 95% prediction intervals (mean plus or minus 1.94 standard deviations) for the models.

3. Results

The curve fitting results are shown in Table 1. For each dataset a similar sigmoidal model gave the best fit. The r^2 coefficients of determination were typically 1% below the highest r^2 obtained for any model, indicating that our biologically plausible choice of sigmoidal model does not lead to significant under-fitting for these data.

Since there were no large-scale variations for any of the datasets, we report a three-parameter cumulative Lorentzian normative model of ovarian volume given by

$$\log_{10}(\text{ovarian volume}) = \frac{a}{\pi}\left(\arctan\left(\frac{\text{age} - b}{c}\right) + \frac{\pi}{2}\right) \quad (1)$$

with height parameter $a = 1.08$ (95% CI 1.02 through 1.13), centre parameter $b = 46.9$ (95% CI 45.4 through 48.3), and width parameter $c = -13.7$ (95% CI −14.6 through −12.9). This model can be interpreted as rapid decline in human ovarian volume from about age 33 to about age 61, with the rate of decline slowing after about age 47. A log-unadjusted version of the model is given in Figure 1, together with intervals in which the ovarian volumes of 68% and 95% of the population are expected to fall (mean plus or minus one or two standard deviations, resp.).

The correlation of mean ovarian volumes against mean NGF population, for ages 25 through 51, is given in Figure 2.

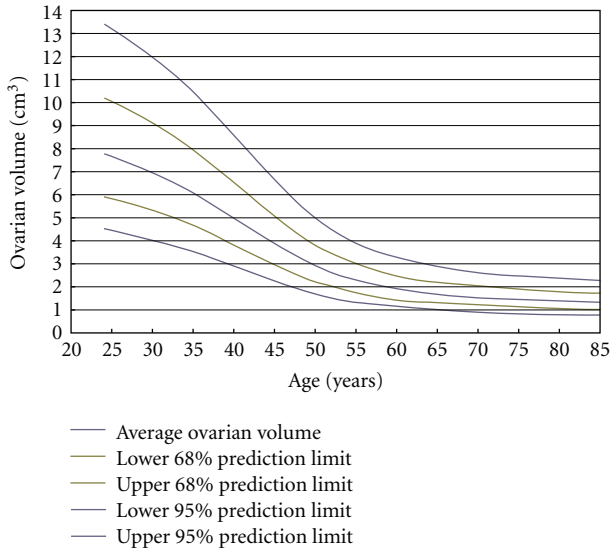

Figure 1: A normative model for the decline in human ovarian volumes from age 25. The centre line is the mean expected value for a given age. 68% of human ovarian volumes calculated at a known age are expected to fall within the lines at 1SD either side of the mean; 95% are expected to fall within the outer lines at 2SD from the mean.

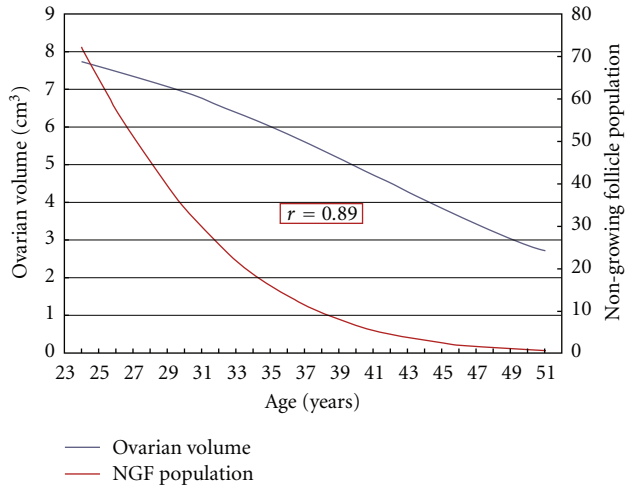

Figure 2: A strong and positive correlation, $r = 0.89$, between log-unadjusted mean ovarian volumes and mean NGF populations given by the Wallace-Kelsey model [1].

We report a strong and positive correlation, $r = 0.89$, for this age range. For log-adjusted values of both ovarian volumes and NGF populations, we report extremely strong and positive correlations, $r = 0.99$, both for mean values and for decile, quartile, and percentual prediction limits. Figure 3 illustrates this for mean values and 95% prediction limits. Using this correlation over all ranges of variation from average, we can infer that a population of 1000 NGFs (i.e., 10^3) at any age represents approximately 3 cm^3 volume (i.e., $10^{0.48}$).

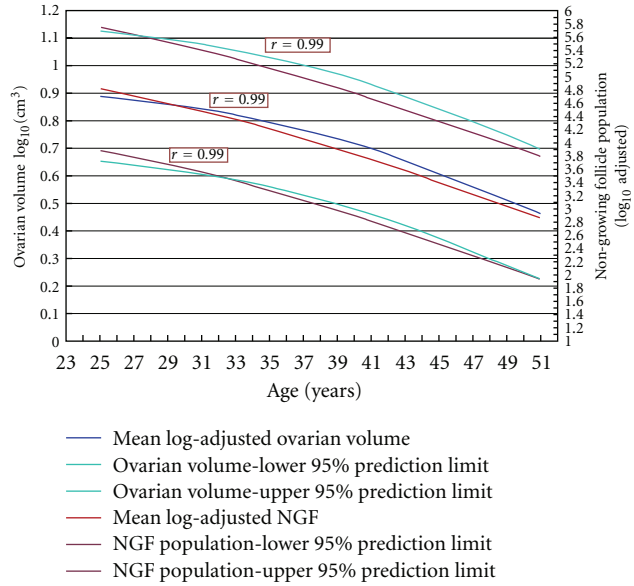

Figure 3: Extremely strong and positive correlations, $r = 0.99$ for each pair of lines, between log-adjusted ovarian volumes and the Wallace-Kelsey model of NGF population [1]. The inner lines are mean values; the outer lines are 95% prediction limits for the respective values. An NGF population of 1,000 (i.e., 10^3) corresponds to an ovarian volume of 3.01 cm^3 (i.e., $10^{0.48}$).

4. Discussion

We have shown that there is a strong and positive correlation between ovarian volume and NGF population in the human ovary. We can therefore hypothesise that small ovaries have reduced numbers of NGFs and furthermore can calculate the number of NGFs in an ovary of known volume. We have also shown that an NGF population of one thousand corresponds to an ovarian volume of 3.01 cm^3.

Our results provide a simple method for assessment of remaining NGF pool for an individual. First obtain an accurate measurement of ovarian volume (by taking the average of two or more transvaginal ultrasound measurements, as set out in [17]). Use the base-10 logarithm of this value and the age of the individual to enter a datapoint on Figure 3. Read the value for this datapoint from the secondary y-axis, and raise 10 to a power of this value. The resulting number is an estimate of the number of NGFs remaining in that ovary.

Previous studies have shown that for women over 34 years of age, ovarian volume correlates strongly with follicular density in cortical tissue [9], and that large ovarian volumes are associated with good assisted reproductive technology outcomes whereas small ovarian volumes are associated with poor outcomes [8]. Our results agree with both sets of findings and also provide the quantitative information needed to say what "large" and "small" ovaries mean for the healthy population. Large (resp., small) ovaries at a known age have volumes greater (resp., less) than 1 SD from the average; very large and small ovaries are more than 2 SD away from average (Figure 1). This study extends and improves a similar study by Wallace and Kelsey [18] that

reported a strong correlation between an earlier model of NGF decline with age and the mean ovarian volumes given by Pavlik et al. In this paper we have used an improved NGF model and have correlated not only average values but also all variations in both ovarian volume and NGF population.

Since data of this size and distribution provide similar goodness-of-fit results for the models tested (Table 1), and since each of our datasets provides the same results as the original paper, we have made the important assumption that the ten generated datasets accurately represent the original dataset [15].

We acknowledge a number of specific study limitations, the most important of which is that no causal link has been shown that explains the high correlation coefficients obtained in this study: it may be the case that a large ovary contains a large number of NGFs (in general) as our results suggest, but there is no direct evidence for this. Bowen et al. [19] have shown that reduced ovarian volumes predict reduced ovarian reserve (in terms of increased FSH) for infertile women, but we know that there are no studies that translate this finding to the fertile (normal) population.

A further limitation is that our ovarian volume model has not been validated: we have not tested how well or poorly the model generalises to unseen data. Indirect evidence for the validity of our model is given by Holm et al. [20]. This study produced a normative model of ovarian volumes for ages ranging from birth to 26 years, with a predicted average volume at age 24 of 7.8 mL. This entirely separate calculation agrees exactly with ours: we also predict an average volume of 7.8 mL at 24 years of age. Our model also agrees in qualitative terms (i.e., curvilinear decline at ages of menopause, followed by linear decline at older ages) with the normative model for postmenopausal ovarian volumes produced by Tepper et al. [21]. Our model reports smaller means and ranges than this study, due either to systematic differences in volume calculation between the two research groups or to the much smaller sample size ($n = 311$) for Tepper et al.

5. Conclusions

We have presented a normative model for human ovarian reserve for ages 25 through 85 and demonstrated that about two thirds of the variation in ovarian volumes for this age range is due to age alone. If our model were to be externally validated, and if it were shown that (in general) larger ovaries contain more NGFs than smaller ones (possibly by inference from a mammalian model), then the strong and positive correlations that we have reported indicate that the remaining follicle pool for individuals can be accurately assessed using ovarian volumes as a surrogate measure. Moreover, our model can be correlated against other models of indirect measures of ovarian reserve, such as the normative model for serum AMH in the healthy population [22, 23]. We speculate that a multivariate model can be derived, involving both endocrine factors and physiological factors (such as ovarian volume and antral follicle count), that will allow the accurate estimation of remaining fertile lifespan for individual women. This would have major implications for the planning of assisted conception cycles and for the preservation of fertility for survivors of cancer earlier in life.

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Clinical Study

Is There a Relationship between Ovarian Epithelial Dysplasia and Infertility?

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Aim. Ovarian epithelial dysplasia was initially described in material from prophylactic oophorectomies performed in patients at genetic risk of ovarian cancer. Similar histopathological abnormalities have been revealed after ovulation stimulation. Since infertility is also a risk factor for ovarian neoplasia, the aim of this study was to study the relationship between infertility and ovarian dysplasia. *Methods.* We blindly reviewed 127 histopathological slides of adnexectomies or ovarian cystectomies according to three groups—an exposed group to ovulation induction ($n = 30$), an infertile group without stimulation ($n = 35$), and a spontaneously fertile control group ($n = 62$)—in order to design an eleven histopathological criteria scoring system. *Results.* The ovarian dysplasia score was significantly higher in exposed group whereas dysplasia score was low in infertile and control groups (resp., 8.21 in exposed group, 3.69 for infertile patients, and 3.62 for the controls). In the subgroup with refractory infertility there was a trend towards a more severe dysplasia score (8.53 in ovulation induction group and 5.1 in infertile group). *Conclusion.* These results raise questions as to the responsibility of drugs used to induce ovulation and/or infertility itself in the genesis of ovarian epithelial dysplasia.

1. Introduction

Histopathological study of material from prophylactic oophorectomies performed for a genetic predisposition of ovarian cancer revealed cytological and architectural abnormalities considered to be precancerous manifestations, and termed "dysplasia" by analogy with the pre-invasive lesions described for the genital tract (vulva, vagina, cervix, endometrium) [1]. Several studies have found similar ovarian dysplasia lesions after stimulation of ovulation in infertile patients, without any indication of their long-term evolution [2, 3]. However, the relationship between ovulation induction and ovarian dysplasia is not obvious because infertility in itself represents a confounding factor [3]. The question is whether the lesions are somehow related to the infertility or are due to the ovulation stimulation.

The aim of this study is to determine the relationship between infertility and ovarian epithelial dysplasia.

2. Methods

2.1. Patients. Using a database covering 1.400 adnexectomies and/or ovarian cystectomies carried out between January 1995 and December 2000, we selected three groups.

Group A: An Exposed Group. Who had adnexectomies and/or ovarian cystectomies after in vitro fertilization using ovulation induction several years later and whose ovaries were

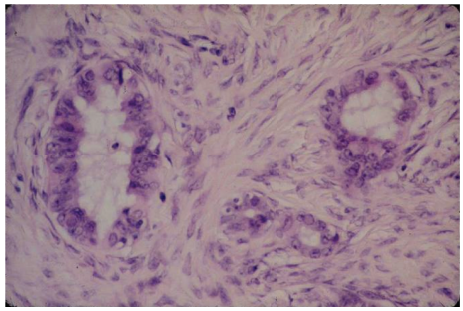

Figure 1: Nuclear abnormalities in ovarian epithelial dysplasia (HES, ×40), from Dr. L. Deligdisch collection.

reported as normal on routine histological examination. We felt it would be interesting to study more particularly those cases in which there will be failure of stimulation. We called this subgroup "refractory infertility."

Group B: An Infertile Group. Without ovulation induction who had adnexectomies and/or ovarian cystectomies prior to any assisted reproductive treatment (ART) technique and whose ovaries were reported as normal on routine histological examination. They did not receive ovulation induction before the surgery. We felt it would be interesting to study more particularly those cases of infertility in which there will be failure of stimulation. We called this sub-group "refractory infertility."

Group C: Control Group. We selected a spontaneously fertile population, with no personal nor family history of gynaecologic neoplasia (breast, ovary, endometrium), who underwent adnexectomy and/or cystectomy for which the histopathological examination concluded that the ovaries showed no sign of cancerous or borderline pathology.

2.2. Histopathological Criteria. Our definition of ovarian atypia was based on previous studies of ovarian dysplasia, that is, dysplasia described in ovaries from patients with a genetic risk (prophylactic oophorectomy for BRCA1/2 mutation) [4–6], in areas that appeared to be "healthy" adjacent to an ovarian cancer [7, 8], in the apparently healthy contralateral ovary in case of unilateral ovarian cancer [9, 10], and in stimulated ovaries [2, 3]. This scoring system (eleven histopathological criteria) was designed in our previous study about the relationship between ovarian dysplasia and ovulation induction [3]:

epithelial pseudostratification,

epithelial proliferation,

surface papillomatosis,

irregular nuclear chromatin pattern (Figure 1),

irregular nuclear contour,

cellular pleiomorphism,

increase in nuclear size

inclusion cysts,

deep epithelial invaginations,

psammoma,

stromal hyperplasia.

In each case, the least normal area was given a score between 0 and 2 (0: normal, 1: moderately abnormal, 2: severely abnormal), whether located on the surface or in an inclusion cyst.

An overall dysplasia score was then obtained for each patient by simply adding the scores for each of the 11 items (total range: 0 to 22).

Morphological studies were processed on 3 micron paraffin sections stained with standard haematoxylin phloxin safran (HPS). The number of sections available for review for each case ranged from 8 to 11 in both study groups.

The histopathology slides were all reexamined blinded by two pathologists who were expert in oncogynaecology. When several slides were available, the one with the highest dysplasia score was retained. Concerning the cystectomies, the slides were re-examined on the one hand to confirm the histopathological diagnosis and on the other to look for associated ovarian tissue in order to establish the dysplasia score. If there was no ovarian tissue the file was excluded.

In the event of obvious differences between the scores established by each pathologist, a further examination was carried out to reach a consensus.

2.3. Statistical Analysis. Our main measurement was the mean dysplasia score. Student's t-test was used to compare the dysplasia score means of both groups.

3. Results

All the included patients are eligible.

30 exposed patients (group A), 35 infertile patients (group B), and 62 fertile controls (group B) were included in the study. There were 18 "refractory infertility"patients in group A and 21 "refractory infertility" patients in group B. The characteristics and the indications of surgery of the three groups are given in Table 1.

Histopathological features of excised material from group A, B, and C were mainly benign cysts (resp., 30 cysts, 32 cysts, and 40 cysts) without cancer or borderline tumor. Histopathological analysis is given in Table 2.

Infertility was female in 70% of cases in group A and 71% of cases in group B, with the following distribution: in group A, ovarian dysovulation 10%, tubal pathology 40%, endometriosis 50% and in group B, ovarian dysovulation 4%, tubal pathology 36%, and endometriosis 60%.

The cytological and architectural abnormalities of the ovarian epithelium described by our score were always assessed in the ovarian tissue. The histopathological abnormalities in both groups are described in Table 3. Histopathological anomalies were always present in group A whereas they were rare in group B.

Based on this data, a mean dysplasia score was determined for both groups: 8.21 for group A, 3.69 for the infertile

Table 1: Characteristics of the study population at the time of surgery.

Variables	Exposed group A $N = 30$	Infertile group B $N = 35$	Control group C $N = 62$
Age (years)	38,5 (29–50)	30.5 (21–43)	42.1 (32–51)
BMI	23,1	23.4	22.6
Surgical indication			
Metrorrhagia	6	0	20
Pelvic pain	18	8	39
Cyst at ultrasound	30	30	35
Hydrosalpinx	0	3	0
Ovarian biopsies	0	3	0
Nulliparity	5	29	0
Parity	1,1 (1–3)	0 (0-1)	2.7 (1–4)
Use of oral contraception	25, or 83%	28, or 80%	55, or 89%
Duration of exposure to oral contraception (months)	40,1 (4–91)	26.5 (0–134)	58.2 (20–180)

Table 2: Histopathological diagnosis on excised tissues.

	Exposed group (group A)	Infertile patients (group B)	Controls (group C)
Pyosalpinx	0	0	5
Hydrosalpinx	0	3*	0
Endometrioma	9	15	12
Serous ovarian cystadenomas	6	3	5
Mucinous ovarian cystadenomas	2	5	6
Ovarian myoma	0	2	5
Follicular ovarian cyst	10	3	9
Haemorrhagic ovarian cyst	3	3	17
Torsion/ischaemia of the adnexa	0	0	3
Ovarian biopsies (ovarian reserve)	0	4*	0

* The 3 patients presenting a hydrosalpinx had several ovarian biopsies.

patients, and 3.62 for the controls. The difference was statistically different between group A and C ($P < 0.0001$). However, there was no significant difference between group B and C ($P = 0.92$), nor were any statistically significant differences found according to the aetiology of infertility.

In the "refractory sterility" group, the dysplasia score was higher in group A than in group B: 8.53 for group A and 5.1 for group B, $P = 0.02$.

An estimate of the study's power is 0.99.

4. Discussion

Ovarian dysplasia was initially described in ovaries with a genetic risk of cancer [1, 4, 5]. By analogy with preinvasive cervical lesions, the generic term "dysplasia" was proposed. The fact that these ovaries could evolve towards malignancy if prophylactic ovariectomy did not take place led to the idea that ovarian epithelial dysplasia was the missing link prior to neoplasia.

More recently similar ovarian lesions described as dysplasia were detected in ovaries stimulated during IVF treatment. Nieto et al. [2] were the first to find significant abnormalities in stimulated ovaries compared to a control population. One of our previous studies confirmed these results (mean dysplasia score 7.64) and also showed that a time effect and a dose effect were probable [3]: histopathologic abnormalities (cf photo) would become more severe and greater in number with an increasing number of stimulation cycles (>3) and after a sufficient lapse of time (over seven years). However it is impossible to predict how they would evolve: the dysplastic profile of stimulated ovaries and ovaries with genetic risk is not the same, which would tend to indicate a different evolution at long term [11, 12]. Animal experiments gave some interesting conclusions. Ovulation in rats has resulted in increased Ki67 expression and dysplastic abnormalities in the ovarian epithelium [13]. Çelik et al. [14] found also a relationship between the number of ovulation-inducted cycles and the severity of ovarian dysplasia: when comparing the rate of ovarian dysplasia in three groups of rats subjected to one, three, and six gonadotrophin cycles, there was a significant trend towards more severe dysplasia as the number of inducted ovulation cycles increased. Ozcan et al. [15] have examined the effects of ovulation induction agents on ovarian epithelium after 6 and 12 cycles: ovarian dysplasia (more severe after 12 cycles) was found to be significant in the ovaries of rats that were given clomiphene citrate, recombinant FSH, and human menopausal gonadotrophin. However no malignant ovarian lesion was found in these three animal studies.

Patients undergoing ovarian stimulation could be at increased risk of ovarian tumors (8.21 versus 3.62): few studies have discussed the possible relationship between exogenous hormones and the risk of developing borderline malignancy of the ovary [11, 12, 16]. Therefore, the discovery of ovarian dysplasia in stimulated ovaries raises the question of the possible responsibility of the treatment used to induce ovulation. So two questions require an answer.

Firstly, is ovarian dysplasia a histopathologic entity, or a simple variant from normal?

Secondly, is infertility or ovulation induction a risk factor for dysplasia, and could it be held responsible for the appearance of dysplastic abnormalities?

(1) One of the major disadvantages of a histopathologic score is that there will be subjectivity when applying it. There is no consensual dysplasia scoring scheme. So we designed an exhaustive scoring system for dysplasia in ovaries at genetic risk and in ovaries in relation with ovulation induction [3, 17, 18]. Although we do not separate cellular changes in inclusion cysts and surface epithelium (ovarian surface epithelial changes are rarer than in inclusion cysts) [19], our histopathological dysplasia score seems to be reproducible (review by several pathologists blinded to clinical data and comparison with control group in order to

Table 3: Comparison of respective frequencies of the 11 histopathologic abnormalities in our dysplasia scoring system.

	Group A $N = 30$	Group B $N = 35$	Group C $N = 62$	Statistical difference P
Epithelial pseudostratification	21 (70%)	11 (31.4%)	17 (27.4%)	$P1 < 0.0001$ $P2 = 0.98$ $P3 = 0.002$
Epithelial proliferation	16 (53.3%)	8 (22.8%)	23 (37%)	$P1 = 0.007$ $P2 = 0.06$ $P3 < 0.0001$
Surface papillomatosis	15 (50%)	7 (20%)	15 (24.1%)	$P1 = 0.009$ $P2 = 0.8$ $P3 < 0.0001$
Irregular nuclear chromatine pattern	13 (43.3%)	7 (20%)	18 (29%)	$P1 = 0.0042$ $P2 = 0.69$ $P3 = 0.001$
Irregular nuclear contour	12 (40%)	11 (31.4%)	12 (19.3%)	$P1 = 0.0012$ $P2 = 0.059$ $P3 = 0.08$
Cellular pleiomorphism	19 (63.3%)	9 (25.7%)	21 (33.8%)	$P1 = 0.0078$ $P2 = 0.32$ $P3 = 0.0045$
Increased size of nucleus	14 (46.6%)	6 (17.1%)	13 (20.9%)	$P1 = 0.0074$ $P2 = 0.86$ $P3 = 0.004$
Inclusion cysts	21 (70%)	15 (42.8%)	31 (50%)	$P1 = 0.032$ $P2 = 0.22$ $P3 = 0.004$
Psammomas	5 (16.6%)	5 (14.2%)	4 (6.4%)	$P1 = 0.017$ $P2 = 0.012$ $P3 = 0.8$
Deep epithelial invaginations	15 (50%)	6 (17.1%)	11 (17.7%)	$P1 < 0.0001$ $P2 = 0.99$ $P3 = 0.002$
Stromal hyperplasia	11 (36.6%)	14 (40%)	10 (16.1%)	$P1 < 0.0001$ $P2 = 0.0013$ $P3 = 0.78$

$P1$: statistical differences between group A and C. $P2$: statistical differences between group B and C. $P3$: statistical differences between group A and B. Statistical analysis by Student's t-test.

validate our dysplasia system in one of our previous studies) [20] and consistent with the literature [4–10]. We have proposed a cut-off in one of our latest studies: an ovarian dysplasia score over than 8 (Se: 60%; Sp: 93.3%) [20]. Digitised morphometric analyses based on the degree of stratification and loss of polarity (by measuring the shortest distance between the nucleus and basal membrane, cellular density), and nuclear pleiomorphism (by measurement of the circumference and surface area of the nucleus) [21], or methods of nuclear karyometry (quantitative analysis of nuclear texture) [22, 23] confirm that dysplasia is indeed a distinct histopathologic entity in its own right. Recent immunohistochemistry and molecular studies gave similar results, validating the concept of "ovarian dysplasia" [24, 25].

(2) Human epidemiological studies following up infertile patients have most often demonstrated an increased risk of ovarian tumour (cancerous or borderline), but the results are contradictory: some blame the infertility itself [26, 27] while others lay the blame more on ovulation inducing agents [28, 29].

Our previous studies revealed significant dysplastic lesions in stimulated ovaries [3]. In the present study, there were significant dysplastic lesions in exposed group whereas there was no increase in the dysplasia score in the infertile patients, which is corroborated by the studies of nulliparous patients by Nieto et al. [2].

Should we therefore conclude that treatments to induce ovulation are responsible for the genesis of dysplasia?

Our results show a significant trend towards dysplasia in case of refractory infertility in group A and B. However other cofactors might be involved. Nieto et al. [30] also explored the prevalence of ovarian cancer in patients who were 1st degree relatives of women treated for infertility (due to anovulation) compared with patients who were 1st degree relatives of spontaneously fertile women: the result was a relative risk of 1.45 (95% IC 0.36–10.55) and above all an additional risk in patients who were 1st degree relatives of patients presenting refractory infertility due to dysovulation (14.8, IC 95% 1.36–160). The authors concluded in a probable "genetic link" [30, 31]. A deletion or mutation in a number of genes which regulate cell cycle and cell death in the ovary could affect both fertility (through regulation of follicle pool) and carcinogenesis (by increasing growth stimilus and/or removing growth inhibition): for example, in vitro studies have proved that mice deficient in LATS1 are infertile and develop ovarian tumours [32]. Deletions of Smad1 and Smad5 lead to infertility and ovarian cancer in mice [33].

So our results could corroborate this genetic theory by showing that this sub-group of patients with refractory infertility would be at risk, with the ovulation inducing drugs possibly acting as dysplasia revealers or accelerators.

Caution is needed when interpreting all this data: this is a retrospective observational study with limited numbers of patients. Infertility is a complex and multifactorial pathology in which many confounding factors interfere (age, parity, breastfeeding, dosage level, duration of contraception, etc.) to the point it is difficult to come to any conclusion about risk factor. Although the study's power is very good (0.99), we cannot draw the conclusion that the ovulation stimulation therapy might always cause ovarian dysplasia. However, we can tell that there is sometimes some histopathological abnormalities in ovaries in relationship with ART and infertility. This is a legitimate and very important question that needs more studies not only to describe the dysplastic lesions more precisely but also to look for them in larger series and for their relationships with ovulation inducing drugs.

5. Conclusion

This study shows a significant level of abnormalities after ovarian stimulation whereas there is no increase in ovarian epithelial dysplasia in infertile patients compared with fertile patients; we can also note a trend towards a higher incidence of dysplastic changes in "refractory infertility."

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

New and Simple Approach for Preventing Postoperative Peritoneal Adhesions: Do not Touch the Peritoneum without Viscous Liquid—A Multivariate Analysis

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Background. Postoperative peritoneal adhesions (PPAs) are an unsolved and serious problem in abdominal surgery. *Method*. Viscous liquids of soybean oil, octyl methoxycinnamate, flax oil, aloe vera gel, and glycerol were used in five experiments, using the same methodology for each. Liquids were applied in the peritoneal cavity before and after mechanical peritoneal trauma. Results were evaluated by multivariate analysis. *Results*. Compared with the control group, macroscopic and microscopic adhesion values before ($P < .001$) and after ($P < .05$) application of viscous liquids significantly reduced PPAs. Values were significantly lower when liquids were applied before rather than after peritoneal trauma ($P < .0001$). *Discussion*. Viscous liquids injected into the peritoneal cavity before or after mechanical peritoneal trauma decrease PPA. Injection before trauma was more effective than after trauma. In surgical practice, PPA formation may be prevented or decreased by covering the peritoneal cavity with an appropriate viscous liquid before abdominal surgery.

1. Introduction

Postoperative peritoneal adhesions (PPAs) are a serious complication experienced by more than three-quarters of patients who undergo abdominal surgery. PPAs are the most common causes of intestinal obstruction, infertility, and abdominal and pelvic pains [1–3]. PPAs also represent a serious economic problem. For example, a multicenter study including all abdominal surgery units in Sweden found that the annual loss due only to small bowel obstruction was more than US$6 million [4]. Various materials and/or techniques have been investigated to prevent or treat PPAs, but to date, no effective solution has been identified.

PPAs are potentially preventable, and several agents that act as barriers between adjacent peritoneal surfaces have been evaluated for prophylaxis [5]. We, the authors, have as a team been for years working on viscous liquids for preventing PPAs. Viscous liquids studied were ricini, zingiber, daphne, orange, sesame, opium, jasmine, chamomile, hazelnut, eucalyptus, mint, myrtle, honey, and soybean oils, as well as octyl methoxycinnamate, flax seed oil, aloe vera gel, and glycerol. Only the last five have been shown to prevent PPAs [6–10]. In addition to determining the effects of these liquids on preexisting PPAs, we assessed their ability to prevent peritoneal trauma. We hypothesized that covering peritoneal surfaces with these viscous liquids may prevent PPA formation by preventing peritoneal trauma.

2. Methods

Experiments were performed between 2006 and 2010 at the Experimental Animal Production and Research Laboratory of Cerrahpasa Medical School, Istanbul University. All animal protocols were approved by the Animal Ethics Committee of Istanbul University, and experiments were performed in accordance with the regulations governing the care and use of laboratory animals outlined in the Declaration of Helsinki.

The viscous liquids used were soybean oil (Soya yagi, Arifoglu Co.), octyl methoxycinnamate (Vazelin sivi, Galenik

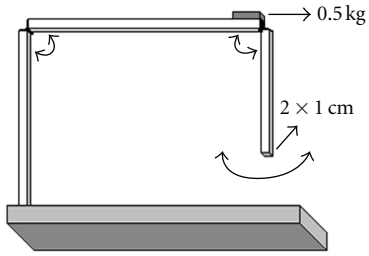

Figure 1: Standart peritoneal adhesion creation apparatus.

Ecza Co.), flax seed oil (Keten yagi, Arifoglu Co.), aloe vera gel (Natural Aloe Vera Gel, Arifoglu Co.), and glycerol (Gliserin, Adora Kimya Co.) [6–10].

We used a total of 160 Wistar outbred female albino rats (mean weight, 190 ± 25 g; mean age, 6.5 months). According to the 3R rule (replacement, refinement, reduction) for experimental animal research, we constructed only one control group for all five experiments, thereby reducing the number of animals by forty.

2.1. Anesthesia Technique. Each rat was anesthetized with 40 mg/kg body weight intramuscular ketamine (Ketalar, Parke Davis and Co., Inc.).

2.2. Adhesion Model. All adhesions were generated using a new apparatus of our own design (Figure 1). The set-up consists of a 20 × 10 cm surgical table with three arms: a stable vertical arm, a moving vertical arm, and a moving horizontal arm. The stable vertical arm works as a shaft and fixes the other two arms onto the surgical table. On the horizontal arm, a 0.5 kg weight is positioned most distal from the shaft. This weight corresponds to the pressure applied by a surgeon's fingertips on the intestinal surface of subjects via manipulation during laparotomies. The horizontal arm transmits the pressure effect of the weight to the lower part of the system by providing up-and-down movements of the system. The other moving arm, with a free pendulum moving along a vertical line, allows application of mechanical trauma (abrasion) on the peritoneal surface. The arm's surface in contact with the peritoneal area (2 × 1 cm) corresponds to the approximate area of a fingertip in contact with a surface (2 cm^2). During the design of this model, a finger of a sterile, powder-free latex glove was placed onto this surface, thus sterilizing this area and increasing the human finger simulation. The anterior face of each rat's cecum was placed under the vertical moving arm; visceral peritoneal trauma was provided by pendulum movement of this arm ten times.

Group 1 ($n = 10$). Control group. The adhesion model was generated.

Group 2 ($n = 50$). In the absence of adhesions, intraperitoneal 0.1 mL of the following sterile viscous liquids was injected interperitoneally in five subgroups:

Group 2.1 ($n = 10$): soybean oil [6],

Group 2.2 ($n = 10$): octyl methoxycinnamate [7],

Group 2.3 ($n = 10$): flax seed oil [8],

Group 2.4 ($n = 10$): aloe vera gel [9],

Group 2.5 ($n = 10$): glycerol [10].

Group 3 ($n = 50$). Prior to the generation of the adhesion model, 0.1 mL sterile viscous liquids was applied to cecum surfaces in five subgroups:

Group 3.1 ($n = 10$): soybean oil [6],

Group 3.2 ($n = 10$): octyl methoxycinnamate [7],

Group 3.3 ($n = 10$): flax seed oil [8],

Group 3.4 ($n = 10$): aloe vera gel [9],

Group 3.5 ($n = 10$): glycerol [10].

Group 4 ($n = 50$). After the adhesion model was generated, cecum surfaces of five subgroups were covered with 0.1 mL sterile viscous liquids:

Group 4.1 ($n = 10$): soybean oil [6],

Group 4.2 ($n = 10$): octyl methoxycinnamate [7],

Group 4.3 ($n = 10$): flax seed oil [8],

Group 4.4 ($n = 10$): aloe vera gel [9],

Group 4.5 ($n = 10$): glycerol [10].

Ten days later, the rats were sacrificed by an overdose of intraperitoneal sodium pentothal (Pentothal Sodium Ampul, Abbott Co.). Laparotomy consisted of a reverse U-shape incision. Without damaging the formed adhesions, the anterior abdomen wall flap was retracted caudally, and adhesions observed in the peritoneal cavity were graded [11] macroscopically according to size and severity (Table 1).

Table 1: Definitions of macroscopic peritoneal adhesion grading system according to size and severity [11].

Grades	Adhesion size	Adhesion severity
0	No adhesion	No adhesion
1	Presence of adhesion in 25% of the area	Spontaneously separating adhesion
2	Presence of adhesion in 50% of the area	Separation of adhesion with traction
3	Whole area covered with adhesion	Separation of adhesion with a sharp dissection

Resected areas of adhesions were fixed in formol and, after dehydration, embedded in paraffin. Cross-sections of 5 mm thickness were prepared, stained with hematoxylin and eosin, and evaluated by light microscopy at a magnification of ×100. All evaluations were performed according to the microscopic fibrosis grading system [11] by a pathologist blinded to methods and groups (Table 2).

The primary outcome measure was a macroscopic adhesion score (average value of adhesion severity and adhesion size grade). The secondary outcome measure was a microscopic fibrosis grade.

Table 2: Definitions of microscopic fibrosis grading system [11].

Grades	Definition
0	No fibrosis (no fibroblasts and/or collagen fibers)
1	Slight fibrosis (few fibroblasts and/or collagen fibers)
2	Median fibrosis (more fibroblasts and/or collagen fibers)
3	Severe fibrosis (lots of fibroblasts and/or collagen fibers)

Table 3: Macroscopic adhesion scores of subgroups 3 (score of control group: 2.90 ± 0.21).

	Prior the generation of adhesions	Difference with control groups
Glycerol	0.00 ± 0.00	$P < .001$
Octyl methoxy	0.40 ± 0.84	$P < .001$
Soy bean oil	0.50 ± 0.71	$P < .001$
Flax oil	0.10 ± 0.32	$P < .001$
Aloe vera gelly	0.55 ± 0.60	$P < .001$
Total	0.31 ± 0.49	$P < .001$

Table 4: Macroscopic adhesion scores of subgroups 4 (score of control group: 2.90 ± 0.21).

	After the generation of adhesions	Difference with control groups
Glycerol	0.30 ± 0.95	$P < .001$
Octyl methoxy	1.80 ± 2.39	$P < .05$
Soy bean oil	1.90 ± 0.94	$P < .05$
Flax oil	0.65 ± 1.80	$P < .01$
Aloe vera gelly	2.60 ± 0.39	$P > .05$
Total	1.45 ± 1.29	$P < .05$

2.3. Statistical Evaluation. All statistical evaluations were performed using the NCSS 2007 program for Windows. Besides standard descriptive statistical calculations (mean and standard deviation), the Kruskal-Wallis test was used to compare groups, and the post hoc Dunn's multiple-comparison test was used to compare subgroups. Statistical significance was defined as $P < .05$.

3. Results

The mean macroscopic adhesion score of the control group (Group 1) was 2.9 ± 0.42 and the mean microscopic adhesion score of this group was 2.8 ± 0.22. Scores of all rats receiving injections of viscous liquids in the absence of adhesions (Group 2) were 0. Rats receiving injections of viscous liquids prior to the generation of adhesions (Group 3) had macroscopic adhesion scores significantly lower than those of the control group (0.31 ± 0.49, $P < .001$, Table 3). Moreover, the mean macroscopic adhesion scores of rats injected with viscous liquids following to the generation of adhesions (Group 4) were lower than those of the control group (1.45 ± 1.29, $P < .05$, Table 4). Group 3 had significantly lower mean macroscopic adhesion scores than Group 4 ($P < .0001$).

Table 5: Microscopic adhesion values of subgroups 3 (value of control group: 2.80 ± 0.42).

	Prior the generation of adhesions	Difference With control groups
Glycerol	0.60 ± 0.51	$P < .01$
Octyl methoxy.	1.30 ± 0.48	$P < .05$
Soy bean oil	0.20 ± 0.42	$P < .001$
Flax oil	0.20 ± 0.42	$P < .001$
Aloe vera gelly	1.20 ± 0.91	$P < .05$
Total	0.50 ± 0.54	$P < .001$

Table 6: Microscopic adhesion values of subgroups 4 (value of control group: 2.80 ± 0.42).

	After the generation of adhesions	Difference with control groups
Glycerol	1.60 ± 0.70	$P < .01$
Octyl mMethoxy.	2.40 ± 0.70	$P > .05$
Soy bean oil	1.50 ± 1.35	$P < .05$
Flax oil	0.40 ± 0.52	$P < .001$
Aloe vera gelly	2.60 ± 0.51	$P > .05$
Total	1.70 ± 0.75	$P < .05$

Overall, the mean microscopic adhesion scores of Group 3 (0.50±0.54, $P < .001$, Table 5) and Group 4 (1.70±0.75, $P < .05$, Table 6) differed significantly from those of the control group (2.80 ± 0.22). Group 3 also had significantly lower mean microscopic adhesion scores than Group 4 ($P < .0001$).

4. Discussion

Since 1999, we have focused on the use of viscous liquids to prevent PPAs and have tested numerous kinds. Our first international manuscript (2002) is related to honey [12]. Because PPAs result from peritoneal trauma [1, 2], two fundamental methods are used to prevent PPA-related complications: the prevention of adhesion formation, and the treatment of adhesions after they formed. The second method is particularly complex because it relates to the wound healing process. This process involves multiple cell populations, the extracellular matrix and the action of soluble mediators such as growth factors, and cytokines, with some steps and molecular actors still unclear [13]. Because of this complexity, we focused on preventing PPAs by covering the peritoneal surface with various viscous liquids that do not negatively affect vital tissues, especially peritoneal mesothelial cells. Covering peritoneal surfaces with viscous liquids may prevent the peritoneum from experiencing mechanical trauma, both by preventing direct contact with the trauma-inducing material and by dispersing any focused pressure via fluid surface tension. We tested our hypothesis using five viscous liquids: soybean oil, octyl methoxycinnamate, flax seed oil, aloe vera gel, and glycerol.

We designed and utilized for the first time the apparatus used to create abrasions in the present study. By means of

this apparatus, we were able to standardize each component of peritoneal trauma. The surface area of trauma was standardized by fixing the size of the surface that would come in contact with the peritoneum (2×1 cm). Trauma location was standardized by making the apparatus fixed and stable so that the pendular movement always affected the same point in each subject, thereby creating abrasions at the same location. The number of incidents of trauma was standardized by using the same number of pendular movements, and pressure was standardized by using a standard force derived from a 500 g weight.

In this extended researches period, we had two major aims: (1) to identify one or more liquids whose specific properties could prevent PPA, and (2) to test our hypothesis that PPAs could be prevented by prevention of peritoneal trauma via covering peritoneal surfaces with approprite viscous liquids. We found that soybean oil, octyl methoxycinnamate, flax seed oil, aloe vera gel, and glycerol were effective in preventing PPAs, with glycerol and flax seed oil being the most effective. Multivariate analysis showed that application of these liquids before or after the generation of peritoneal trauma significantly decreased PPAs. Moreover, application before trauma was significantly more effective than application after trauma.

Results of macroscopic evaluation relate to the clinical effectiveness of this new approach. Results of microscopic evaluation either support the macroscopic results or explain the mechanism of PPAs. In order to prevent peritoneal trauma directly (mechanically), histopathologic fibrosis values of the before-application groups were statistically differently diminished than those of control and after-application groups.

In conclusion, we have shown that application of viscous liquids to the peritoneal cavity before or after mechanical peritoneal trauma decreased PPA formation, with application before trauma being more effective. It is important to note that our findings result from experiments conducted in animals and need to be evaluated in humans. It is important point that our results are conducted in animals and need to be evaluated in humans. Application of a viscous liquid before peritoneal trauma is the new, simple and also cost-effective approach to preventing of PPA formation. Thus, in surgical practice, PPA formation may be prevented or decreased by applying an appropriate viscous liquid to the entire intraperitoneal cavity or area of manipulation before laparotomy/laparoscopy. Future studies on the use of viscous liquids with no negative effects on vital tissues, especially peritoneal mesothelial cells with different application modalities, are needed for more reliable results.

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Clinical Study

Dosage Optimization for Letrozole Treatment in Clomiphene-Resistant Patients with Polycystic Ovary Syndrome: A Prospective Interventional Study

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Objective. Dose adjustment for induction of ovulation is one of the most important problem. *Methods*. In a prospective interventional study, 44 clomiphene-resistant infertile patients (113 cycles) were selected from the Abolfazl Infertility Clinic of Bushehr University of Medical Sciences. Letrozole was given orally in a dose of 2.5 mg, 5 mg, and 7.5 mg, respectively. If the patient displayed no response, the dosage was increased. *Results*. In this patients ovulation occurred in 50 cycles (44.24%), clinical pregnancy rate according to number of cycles was 23.89% (27 of 113 cycles) and according to the number of patients was 61.36% (27 of 44 patients). In the 2.5, 5, and 7.5 groups, follicles occurred in 22.9%, 42.1%, and 85.18% of cycles, and pregnancy rate was 14.58%, 28.94% and, 33.33%, respectively. *Conclusions*. It is better to administer Letrozole at a lower dosage to prevent complications and increase the dose based on sonographic results antral follicular count, anti-Müllerian hormone, LH/FSH, and estradiol.

1. Introduction

Letrozole is an effective treatment for anovulatory infertile women. Letrozole appears not to have any adverse effects on the endometrium which is frequently associated with clomiphene citrate during ovulation induction [1, 2]. Many researchers have tried letrozole for ovulation induction in different methods [3–9]. Letrozole induced fewer mature follicles that can decrease multiple-pregnancy rate and risk of ovarian hyperstimulation syndrome. Therefore, the letrozole as the first-line drug of ovulation-induction agents in polycystic ovarian syndrome (PCOS) patients can be acceptable [10]. Segawa et al. have accepted letrozole and clomiphene to have the same effect in pregnancy outcome in PCOS patients although letrozole is not toxic and does not have any significant congenital anomaly associations [11–13]. In this study we have recommended letrozole in serial doses of 2.5, 5, and 7.5 mg in each cycle in clomiphene citrate-resistant infertile women with PCOS. The main interest of this paper is the assessment of efficacy and complication of letrozole in follicular size and number, pregnancy rate, abortion, endometrial thickness, and cumulative pregnancy rates in a clinical trial of clomiphene citrate-resistant patients.

2. Materials and Methods

In this prospective intervention, we studied Abolfazle Clinic's outpatients of the Bushehr University of Medical Sciences in Iran who referred between January 1, 2008, and December 30, 2010. The study was approved by the institutional ethics committee of the Bushehr University of Medical Sciences, and all patients were required to provide written informed consent before the study commenced. Inclusion criteria are as follows. The study group consisted of polycystic ovarian syndrom (PCOS) patients diagnosed according to the Rotterdam criteria [1]. We defined clomiphene citrate (Clomid, Iran Hormone, Tehran, Iran) resistance as failure to achieve adequate follicular maturation after consumption of 3 cycles

of cc at 150 mg/day, determined by serial estradiol monitoring and sonography [1]. Patients resistant to clomiphene citrate became candidates for letrozole (Femara, Novartis, QC, Canada) consumption at the step-up of the protocol. Patients information was recorded in Table 1. Exclusion criteria were abnormal thyroid function test, hyperprolactinoma, galactorrhea, male-factor infertility, tubal and uterine causes of infertility (hysterosalpingography), abnormal response in progesterone challenge test which implies no endogenous estrogen production, FSH > 10, poor patient compliance or complications with treatment. The required information was gathered through demographic and infertility interviews using an information list. Each patient was followed for 6 sessions. Estradiol levels (E2) were measured using the ELISA method by RAYTO set and DIAPLUS kit before the administration of HCG. Serial sonography (Hoda 4000, Japan) was conducted from the 10th day of menstruation and depended on follicular size (18–24 mm) from which HCG (human chorionic gonadotropin, Pregnyl, Organon, Oss, The Netherlands) was administered. There were 3 steps in which we prescribed letrozole. In all cases, daily administration began on the 3rd day of the menstrual cycle through to the 7th day (totalling 5 days). In the first step we prescribed letrozole at a dose of 2.5 mg (one tablet). Normal follicular size and endometrial thickness were considered 18–24 mm and 6 mm or more, respectively, [1]. If the follicle was deemed not acceptable, the dose of letrozole was increased at the next cycle. At the second and third steps we prescribed letrozole at a dose of 5 mg daily and 7.5 mg/day, respectively, and according to patient's response, repeated the same dose. In the current study we tested the hypothesis that prescribing letrozole as an ovulation induction agent in infertile women would increase pregnancy rate, ovulation, and follicle number. The primary outcome measure was normal follicular size, and the secondary outcome measure was the clinical pregnancy. Clinical pregnancy was considered as the presence of a gestational sac with fetal heart activity. Letrozole tablets were prescribed by an experienced nurse who thoroughly explained the method of use to the patients. Sonography was done by an experienced radiologist. Statistical analysis was performed by the Statistical Package for Social Science version 11.5 for windows (SPSS Inc., Chicago, IL, USA). The data was analyzed by student's t-test and chi-squared test for linear trend and comparing proportions. A P value of <0.05 was considered to be statistically significant.

3. Results

Demographic information was described in Table 1. These 44 patients had received Clomid in 3 sessions (150 mg), and their sonography results did not show any dominant follicle in serial sonography and were known as clomiphene citrate resistant. According to the flow chart (Figure 1), treatment was started. Number of follicles was summarized in Table 2. There was a significant linear relationship between letrozole dosage and follicular number (χ^2 for linear trend: 25.6, $P < 0.0001$), and this trend was significant in each step (χ^2 for linear trend: 11.48, $P = 0.005$) (Table 2). Complications were presented in Table 3. There was a significant linear trend between Letrozole dosage and its complications (χ^2 for linear trend: 6.38, $P < 0.011$) (Table 3). Characteristic of letrozole cycle was showed in Table 4.

Table 1: Patient and cycle characteristics.

Characteristics	Mean ± SD	Range
Age (years)	26.67 ± 5.14	18–39
BMI (kg/m^2)	25.8 ± 2.4	19.3–29.85
Duration of infertility (years)	3.5 ± 0.82	2–8
Basal FSH (mIU/mL)	5.95 ± 1.48	<10
Basal LH (mIU/mL)	7.12 ± 2.2	<12
Basal Esteradiol (pg/mL)	45.98 ± 21.33	25–75
Basal TSH (μIU/mL).	2.18 ± 1.4	0.88–4
Basal prolactin (ng/mL)	20.14 ± 2.89	<24
DHEAS (Ug/dL)	220 ± 130	35–430
Testosterone (ng/mL)	0.48 ± 0.3	0.1–1.8

Table 2: Number of follicles in step-up protocol.

Dosage	One follicle	Multifollicles
Letrozole 2.5 mg	10 (83.33%)	2 (16.66%)
Letrozole 5 mg	11 (68.75%)	5 (31.25%)
Letrozole 7.5 mg	15 (65.21%)	8 (34.78%)

χ^2 for linear trend: 25.6, $P < 0.0001$.

Table 3: Complication of letrozole in step-up protocol.

Dosage	AUB	Ovarian cyct
Letrozole 2.5 mg	2.08%	—
Letrozole 5 mg	2.63%	2.63%
Letrozole 7.5 mg	14.61%	3.7%

χ^2 for linear trend: 6.38, $P < 0.011$.

Table 4: characteristics of letrozole cycles.

Variable	Letrozole groups Mean ± SD	Letrozole total cycle
Follicular development (mm)	20.3 ± 1.89	113
Serum E2 one day of HCG (pg/mL)	120 ± 15.5	113
Pregnancy/cycles	27 (23.89%)	113
Pregnancy/total patients	27 (61.36%)	113

4. Discussion

In this study clomiphene citrate-resistant patient had different response to letrozole in a way that 44.24% of the cycles had normal follicles and 23.89% of them resulted in pregnancy. Increasing the dosage can improve the chance of ovulation and pregnancy. Only 7 (15.9%) patients with 2.5 mg letrozole daily became pregnant while 11 patients were pregnant by increasing the dosage to 5 mg, and, among those who did not respond to these dosages, 9 patients became pregnant by increasing it to 7.5 mg. About 61.36% of the patients became pregnant with letrozole although letrozole is

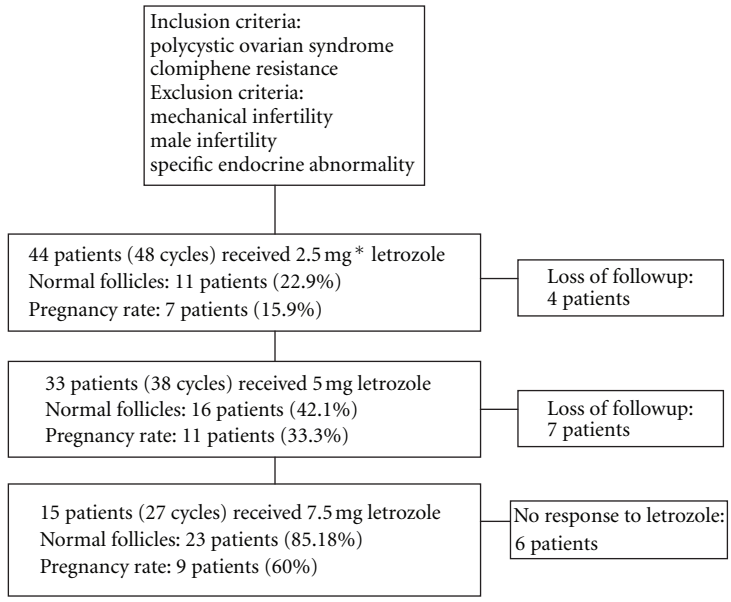

Figure 1: Flow chart of patient's treatment. *mg (milligram).

more expensive than clomid but cheaper than gonadotropins and so is more cost effective. The metformin-clomiphene citrate combination was seen to increase the ovulatory and pregnancy rate when compared with CC alone. Metformin increased the ovulatory rate in clomiphene citrate failures, also implying increased sensitivity to clomiphene citrate [14]. Akbary-Asbagh et al. and Begum et al. suggest letrozole as an effective treatment for clomiphene citrate-resistant (PCOS) patients [15, 16]. Two of 113 cycles resulted in twins; increasing the dosage improved the chance of two follicles in one cycle. In the first step, like Akbary-Asbagh et al. and Begum et al. study [15, 16], all the cycles had one follicle, but 31.25% and 30.43% of cycles had two follicles in 5 mg and 7.5 mg Letrozole regimen, respectively, even one patient had 3 follicles in a cycle. In Mitwally et al.'s study, for all PCO patients with a serial increase of letrozole dosage (set-up protocol) the same results were confirmed which may be due to the long-term inhibition of estrogen levels (E2) [17]. When a patient responds to clomiphene citrate, the E2 will increase considerably which will be greater than when she receives Letrozole [2, 4]. Because of clomiphene citrate resistance, E2 levels do not increase by clomiphene citrate consumption, and beside ovulation its level will be less than the acceptable level in letrozole groups [6]. Basal testosterone level is a good marker for pregnancy outcome and quantity of dominant follicles on HCG day in women with reduced ovarian reserve but not in women with normal range of serum FSH [18]. Letrozole is an effective ovulation induction drug in elevated-BMI women [19]. We had not any correlation between BMI, basal testosterone, LH/FSH, and number of mature follicle because according to inclusion criteria all of the patients had normal testosterone level, BMI, and LH/FSH ratio. The chance of AUB increased from 2.08% with 2.5 mg letrozole daily to 2.63% with 5 mg daily and 14.8% in the 7.5 mg regimen. No change was found in endometrial thickness according to the Cortines study [6]. So AUB could be due to a decrease in estrogen levels. Given these findings, it is required that more investigations be conducted with estrogen drugs like conjugated estrogens which can prevent AUB in cases with additional letrozole dosage. Letrozole can block estrogen (E) production, consequential in decreased negative feedback of E on pituitary for FSH secretion. Bentov et al., prove that a ratio of cycle day 7 to cycle day 3 postletrozole FSH level >1.5 is related with poor ovarian response. Letrozole challenge test can be a prediction of ovarian response [20]. We suggest that higher pregnancy rates during letrozole treatment can be achieved if antral follicular count, anti-Müllerian hormone, LH/FSH, and estradiol are checked and good patient selection is done [1]. The good pregnancy results and low multiple gestation rate of 2.5 mg letrozole for induction of ovulation is hopeful for letrozole user as a first-line drug [12]. The chances of multigestational pregnancy increases by increasing the number of follicles in a cycle which implies an appropriate response to letrozole. Cumulative pregnancy rates were considerable in clomiphene citrate resistant patients, but the pregnancy rate was not significantly different between the 2.5, 5, and 7.5 mg regimens while complications (multigestational pregnancy risk, irregular bleeding, and ovarian cyst) increased by dosage. Delivery rate was 25 live birth/cycles (22.12%) due to 2 abortions during the study.

5. Conclusion

Finally it is concluded that in Clomid-resistant patients, it is better to start letrozole with the lower dosage to prevent more complications and increase dosage based on the sonographic results, antral follicular count, anti-Müllerian hormone, LH/FSH, and estradiol.

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Review Article

Endometrial Stem Cells and Reproduction

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Abnormal endometrial function remains a significant cause of implantation failure, recurrent pregnancy loss, and other pathologies responsible for female infertility. The development of novel therapies to treat infertility due to endometrial dysfunction requires an understanding of the latest advancements in endometrial cell biology, such as the role of endometrial stem cells. The remarkable regenerative capacity of the human endometrium is absolutely essential for successful reproduction and likely requires a population of stem cells in the endometrium. The purpose of this review is to provide an introduction to some of the newest concepts in endometrial stem cell biology.

1. Introduction

Successful reproduction in mammalian females requires a precisely timed and complex interaction between the hypothalamic-pituitary-ovarian (HPO) axis and the uterine endometrium. Although significant advances in reproductive medicine have achieved effective therapies for abnormalities in the HPO axis, abnormalities in endometrial function remain poorly understood. Abnormal endometrial function remains a significant cause of implantation failure, recurrent pregnancy loss, and other pathologies which lead to female infertility. Development of effective therapy for infertility due to endometrial dysfunction requires enhanced understanding of the latest advancements in endometrial cell biology such as the role of stem cells. The purpose of this review is to provide an introductory guide to understanding some of the newest concepts in endometrial stem cell biology.

The human endometrium, derived from the mucosal lining of the fused mesodermal (paramesonephric) tubes (the müllerian ducts) during embryogenesis, is a dynamic tissue. It is comprised of two major zones: (1) the functionalis, a transient layer containing glands extending from the surface (luminal) epithelium as well as the supportive stroma, and (2) the basalis, comprised of the basal region of the glands, stroma, supporting vasculature, and lymphoid aggregates. Although various leukocyte cell types populate the endometrial stroma, including T and B cells, mast cells, macrophages, and neutrophils, the majority of the leukocytes populating the decidualized endometrium of the late secretory phase and early pregnancy are phenotypically unique, tissue-specific lymphocytes known as uterine natural killer (uNK) cells [1]. These cells play a key role in the establishment and maintenance of early pregnancy [2]. Yet, despite their well-documented importance to successful reproduction, their lineage and origin remain unknown.

Human endometrium is unique in its temporally regulated processes of cellular proliferation, differentiation, and shedding of the functionalis layer with each menstrual cycle. The endometrium has the distinctive ability to undergo physiologic angiogenesis in order to facilitate implantation, as well as to regenerate an entirely new functionalis layer following each menses. This remarkable regenerative capacity is essential for successful human reproduction. Although the mechanisms which allow for it remain poorly understood, it is likely to require a uterine stem cell population [3–6].

Somatic stem cells have been identified in many tissue types, including intestine, skin, and bone marrow, and are crucial for physiologic tissue renewal and regeneration after injury [7]. Somatic stem cells are undifferentiated cells, defined by their ability to both self-renew and differentiate into

mature progeny cells of a given tissue type. Evidence exists to support the presence of a resident stem cell population in the uterus, but the location and origin of these cells is unknown [3–6, 8, 9]. A number of possibilities exist as to the origin of endometrial stem cells [4, 6, 9]: (1) they may represent fetal epithelial and mesenchymal stem cells which remain in the adult endometrium and continue to replicate in adulthood, (2) they may represent circulating stem cells arising from a hematogenous source (such as the bone marrow) that seeds the endometrium either periodically or in response to injury, or (3) they may represent a combination of the above.

The hypothesis that cyclic regeneration of the endometrium is mediated via a resident stem/progenitor cell population in the uterus was originally based on early experimental studies in rhesus monkeys, which revealed that removal of all visible tissue via endometriectomy was followed by, after a short delay, reconstruction of a new endometrium [10]. Clinical observations that some women who underwent complete endometrial ablation later developed areas of functional endometrial tissue [11] further supported this hypothesis. Subsequent studies by Padykula et al. [12, 13] provided additional, albeit indirect, evidence of the existence of an endometrial stem cell compartment. Using [^{3}H]thymidine incorporation during the natural menstrual cycle of the rhesus monkey, these investigators demonstrated that the primate endometrium possessed a germinal compartment localized to the lower basalis, in which high epithelial activity persisted postovulation and appeared to escape inhibition by progesterone. This compartment persisted after menses and was postulated to give rise to the transient functionalis layer. These studies, performed in a primate model with menstrual cycles identical to that of the human, provided the basis for the hypothesis that the basalis is the location of a stem cell population in the human endometrium [12, 13].

2. Endometrial Stem Cells: Evidence for Their Existence

Adult stem cells in the endometrium are difficult to identify because they constitute very small populations of cells, and because cell surface markers specific for adult stem cells have not been definitively characterized [4]. Studies which provide indirect evidence for the existence of endometrial stem cells do so by characterizing cell populations in the endometrium which exhibit the functional properties of stem cells. These properties include clonogenicity, proliferative potential, and capacity for differentiation into one or more lineages [4]. Such functional assays provide evidence for the existence of adult stem cells but unfortunately do not allow for localization of the cells within a given tissue.

Clonogenicity, defined as the ability of a single cell to produce a colony when seeded at very low densities, was demonstrated in human endometrium for the first time in 2004 [14]. Using purified single cell suspensions dispersed from hysterectomy specimens, Chan et al. identified small populations of epithelial (0.22%) and stromal cells (1.25%) in human endometrium that possessed clonogenic activity [14]. Large colonies containing >4000 cells were rare and postula-ted to be initiated by stem/progenitor cells, whereas the more common small colonies were postulated to be initiated by more mature transit amplifying cells. A more recent study by these investigators [15] demonstrated that, for both epithelial and stromal cells, clonogenicity did not vary by cycle phase or between active (cycling) and inactive endometrium. The finding, however, of clonogenic cells in inactive endometrium further supports the existence of an endometrial stem cell niche in the basalis, as inactive endometrium is predominantly basalis and lacks functionalis [15].

Other properties evaluated in characterization of an endometrial stem cell population include the capacity for uni- or multilineage differentiation. The differentiation potential of candidate stem cells is evaluated after culturing the cells in differentiation-induction media, then analyzing the cells for expression of phenotypic differentiation markers. Gargett et al. [16] evaluated proliferative and differentiation potential of clonogenic human endometrial cells. Proliferative potential was assessed by serially passaging individual epithelial and stromal colony forming units (CFU) until senescence; large CFU underwent >30 population doublings before senescence, indicating their high proliferative potential characteristic of stem/progenitor cells. Single epithelial CFU differentiated into mature glands in vitro, and large secondary stromal clones demonstrated multipotency as their progeny differentiated into smooth muscle cells, adipocytes, chondrocytes, and osteoblasts, when cultured in typical differentiation-induction media. Thus, both epithelial progenitor cell and multipotent mesenchymal stem cell- (MSC-) like populations were identified in human endometrium. MSCs are multipotent cells located in the bone marrow and multiple other tissues and have the ability to differentiate into cells of multiple mesoderm-derived lineages, such as bone, cartilage, muscle, and adipose tissue [17]. Similarly, Wolff et al. [18] demonstrated the presence of multipotent cells in human endometrium by inducing chondrogenic differentiation of a subpopulation of endometrial stromal cells in vitro, identified by expression of type II collagen and sulfated glycosaminoglycans, characteristic of chondrocytes. It must be recognized, however, that although these studies provide evidence for the existence of putative endometrial epithelial and stromal stem cells, differentiation studies do not rule out the possibility of dedifferentiation of mature stromal cells in the presence of differentiation-induction media, a major limitation of the existing human in vitro differentiation studies.

Another approach used by multiple investigators to identify and characterize stem cells in the human endometrium is the isolation of cells with the "side population" phenotype. Side population cells are characterized by their ability to exclude the DNA-binding dye Hoechst 33343 by expressing ATP-binding cassette transporter proteins [19] and exhibit the properties of adult stem cells including long-term proliferative potential and differentiation into mature tissue-specific cell types. This method has been used to identify putative stem cell populations in multiple tissues, including bone marrow [19], liver [20], mammary gland [21], skin [22], and kidney [23]. More recently, this method has been utilized for the identification and characterization of stem cells in human endometrium. Side population cells isolated

from human endometrium have been demonstrated to display long-term proliferative properties as well as differentiation into mature endometrial glandular epithelial, stromal, and endothelial cells both in vitro [24, 25] and in vivo in immunodeficient mouse models [25–27]. Additional studies have reported the ability of endometrial side population cells to differentiate in vitro into adipocytes and osteocytes, supporting a mesenchymal origin of these cells [26, 28]. Taken together, the growing amount of literature utilizing this technique, albeit limited to few laboratories worldwide, supports the hypothesis that side population cells isolated from human endometrium are indeed somatic stem cells, and that these cells are a source of mature endometrial cell types.

Although phenotypic markers specific to endometrial stem cells have yet to be definitively identified, Schwab and Gargett [29] demonstrated that the perivascular markers CD146 and PDGF-Rβ enabled isolation of stromal cells from human endometrium which exhibit phenotypic and functional properties of mesenchymal stem cells (MSCs). The investigators then used immunohistochemistry to localize these cells to perivascular areas of the basalis and functionalis. They hypothesized that these endometrial "MSC-like" cells may contribute to cyclic regeneration of the endometrium and further postulated that they may play a role in the pathogenesis of diseases such as endometriosis and adenomyosis [29].

3. Localization of Endometrial Stem Cells: Mouse Models

A major limitation of the human studies performed to date is their inability to definitively identify the origin and location of candidate stem cells in the endometrium. Investigators have thus turned to mouse models, using a technique which takes advantage of the quiescent nature of stem cells. In this approach, known as the label-retaining cell (LRC) approach, animals are injected with a thymidine analogue (bromodeoxyuridine or BrdU) which becomes incorporated into genomic DNA during the replication phase of mitosis, and the tissue of interest is examined for cells which retain this label after a prolonged chase period due to infrequent cell divisions (characteristic of somatic stem cells). Using this technique, Chan and Gargett [30] identified 3% of epithelial cells (predominantly luminal) and 6% of stromal cells which were adjacent to the luminal epithelium at the endometrial-myometrial junction, as LRC. A subsequent study [31] detected stromal LRC in a similar location, but none in the epithelial compartment, after a prolonged chase period. A more recent study [32] did not evaluate the stromal compartment but identified epithelial LRCs predominantly in the glandular epithelium. Thus, the existing data on the location of the endometrial stem cell niche in a mouse model are unclear as to cell type(s) and require further study, but they support the existence of a small population of uterine stem cells which are a likely source of regenerative endometrium.

4. The Bone Marrow as a Source of Endometrial Stem Cells

Mesenchymal stem cells (MSCs) from the bone marrow have been demonstrated to differentiate into mature cell types of various nonhematopoietic organs including liver, skeletal muscle, brain, and skin [33]. Recent data from a limited number of investigators support the concept that bone marrow is an important contributor of stem cells to the endometrium. Three independent investigators have identified human endometrial stromal, glandular, and/or endothelial cells of donor bone marrow origin in a total of 8 recipients of bone marrow transplantation from either HLA-mismatched [34] or male [35, 36] donors. These findings were provocative, supporting the ability of bone marrow-derived cells to generate endometrial cells de novo. To date, only three independent laboratories have reported the use of a murine bone marrow transplant model to determine whether bone marrow-derived cells give rise to endometrial parenchymal cell types. Du and Taylor [37] identified Y chromosome-positive endometrial epithelial and stromal cells in female recipients of bone marrow transplant from male donors six months post transplant. A similar approach was used by Mints and colleagues [36], who performed murine bone marrow transplantation using male donors and identified endothelial cells of donor origin in recipient endometria 40 days post transplant. Bratincsák et al. [38] created a transgenic mouse model in which all $CD45^+$ cells coexpressed Green Fluorescent Protein and demonstrated that $CD45^+$ hematopoietic progenitor cells contributed to uterine epithelium. Collectively, these data, albeit limited, support the hypothesis that the bone marrow is an important source of endometrial stem cells, exhibiting the capacity to differentiate into parenchymal and endothelial cell types.

Only one laboratory to date has previously tested the in vitro capacity of human bone marrow-derived cells to differentiate into mature endometrium. Multipotent mesenchymal stem-like cells which express cell surface markers typical of bone marrow MSCs have been identified in human endometrium [16, 29]. Given this line of evidence, Aghajanova et al. [39] recently tested the ability of human bone marrow-derived MSCs to differentiate into endometrial decidual cells. Human neonatal dermal fibroblasts were used as a control to determine whether mature fibroblasts could transdifferentiate under the same culture conditions. After 14 days of treatment with 8-bromo-cyclic adenosine monophosphate (a potent decidualizing agent of human endometrial stromal cells), human bone marrow-derived MSCs (but not dermal fibroblasts) displayed morphologic features characteristic of decidual cells and expressed the classical markers of decidualization, prolactin and IGFBP-1. These studies further support the bone marrow as a potential precursor of endometrial cells. Whether another major population of bone marrow-derived cells, lymphohematopoietic stem cells (precursors of all hematopoietic lineages), are a potential progenitor of human endometrial parenchymal or immune cell types remains to be investigated. Nonetheless, the concept of bone marrow-derived endometrial progenitor cells is a provocative one and bears significance not only in mechanisms underlying normal endometrial physiology but also in disorders of endometrial proliferation, such as endometriosis, endometrial hyperplasia, and endometrial carcinoma. Resident (as opposed to bone marrow-derived) epithelial and/or "MSC-like" stem cells may also contribute to such diseases

[4, 6, 8, 40]. Furthermore, the bone marrow as a source of endometrial cells has therapeutic implications in the treatment of diseases such as Asherman's syndrome, or poorly understood disorders of implantation, important causes of female infertility.

5. Uterine Natural Killer (uNK) Cells

Another major limitation of the uterine stem cell studies performed to date is that the cellular sources of endometrial epithelium and stroma have been the predominant focus of study, with limited attention to the cellular origin of a critical cell type in endometrial function: the uterine natural killer (uNK) cell. Phenotypically and functionally different from peripheral NK cells, these cells are crucial in the establishment and maintenance of early pregnancy [2]. They become abundant in the human uterus 3–5 days post ovulation and by late secretory phase account for at least 30% of the endometrial stroma [41]. Human in vitro studies provide evidence for the ability of uNK cells to promote placental vascular growth and decidualization via production of chemokines and angiogenic factors [2]. Similarly, murine studies indicate that uNK cells are essential for induction of spiral arteries, mediated via their production of IFNγ [42]. Indeed, the finding that uNK-deficient mice exhibit compromised placentation and fetal growth [43] supports a critical role of the uNK cell in reproductive function. However, despite their importance in reproductive function, the cellular origin of uNK cells remains unclear.

A small number of studies have been performed utilizing murine bone marrow transplant models and human in vitro studies, which support the bone marrow as a source of uNK cells. Peel et al. [44], who performed some of the earliest studies which provided evidence for bone marrow origin of uNK cells, utilized a rat-to-mouse bone marrow transplant model to identify donor-derived uNK cells in deciduomata of pseudopregnant recipients. Guimond et al. [45] subsequently demonstrated in murine studies that bone marrow transplantation from NK cell-competent to NK cell-deficient mice led to restoration of the uNK cell population in recipients as well as restoration of normal decidualization/placentation and fetal viability. Further evidence for a nonuterine source of uNK cells has been provided by Chantakru et al. [46], who demonstrated that grafting of uterine segments from NK cell-competent mice into uNK cell-deficient mice revealed the absence of uNK cells in the decidualized grafts. However, transfer of cells from secondary lymphoid tissues (thymus, spleen, peripheral and mesenteric lymph nodes) reconstituted the uNK cell population in recipients. More recently, Vacca et al. [47] demonstrated the ability of $CD34^+$ cells (the phenotype of bone marrow-derived hematopoietic precursors) present in human decidua to differentiate in vitro into uNK cells, either in the presence of certain growth factors or in coculture with decidual stromal cells. These uNK cells were functional (producing IL-8 and IL-22, characteristic products of human uNK cells) and expressed phenotypic cell surface markers of human uNK cells ($CD56^{bright}/CD16^-$). However, additional studies, particularly in human tissues, are clearly necessary in order to clarify whether or not the bone marrow represents a significant source of uNK cell precursors.

In summary, additional studies are necessary to provide us with an enhanced understanding of the capabilities and limitations of endometrial stem cells to determine the potential therapeutic uses for these cells.

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Review Article

The Ethical, Legal, and Social Issues Impacted by Modern Assisted Reproductive Technologies

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Background. While assisted reproductive technology (ART), including *in vitro* fertilization has given hope to millions of couples suffering from infertility, it has also introduced countless ethical, legal, and social challenges. The objective of this paper is to identify the aspects of ART that are most relevant to present-day society and discuss the multiple ethical, legal, and social challenges inherent to this technology. *Scope of Review*. This paper evaluates some of the most visible and challenging topics in the field of ART and outlines the ethical, legal, and social challenges they introduce. *Major Conclusions*. ART has resulted in a tectonic shift in the way physicians and the general population perceive infertility and ethics. In the coming years, advancing technology is likely to exacerbate ethical, legal, and social concerns associated with ART. ART is directly challenging society to reevaluate the way in which human life, social justice and equality, and claims to genetic offspring are viewed. Furthermore, these issues will force legal systems to modify existing laws to accommodate the unique challenges created by ART. Society has a responsibility to ensure that the advances achieved through ART are implemented in a socially responsible manner.

1. Introduction

ART is currently a commonplace technology that has successfully treated millions of infertile couples the world over. However, the explosion of this technology has introduced a myriad of new social, ethical, and legal challenges. This paper evaluates some of the most visible and challenging topics in the field of ART and outlines the ethical, legal and social challenges they introduce.

2. Scope of ART Utilization

Infertility has traditionally been an area of medicine in which physicians had limited means to help their patients. The landscape of this field changed dramatically with the announcement of the birth of Louise Brown in 1978 through *in vitro* fertilization (IVF). This historic moment was eloquently encapsulated by Howard Jones who observed "Eleven forty-seven p.m. Tuesday, July 25, 1978, was surely a unique moment in the life of Patrick Steptoe. This was the hour and minute he delivered Louise Brown, the world's first baby, meticulously, lovingly, and aseptically conceived in the laboratory, but popularly referred to as the world's first test tube baby" [1]. The importance of this birth to scientists, clinicians, and most particularly infertile patients throughout the world cannot be overstated. In several short decades, IVF has exploded in availability and use throughout the world.

Worldwide, more than 70 million couples are afflicted with infertility [2]. Since the first successful IVF procedure in 1978 [3], the use of this and related technologies has expanded to become commonplace around the globe. Over the past decade, the use of ART services has increased at a rate of 5–10% annually [4, 5].

In 1996, approximately 60,000 IVF cycles were initiated in the United States with approximately 17,000 clinical pregnancies and 14,000 live births [6]. Currently, IVF accounts for approximately 1% of all live births in the United States [6]. As of 2009, 3.4 million children have been born worldwide after ART treatment, and ART utilization is

currently increasing at a rate of 5–10% annually in developed countries [4].

3. Reporting Regulations

The widespread use of this technology throughout the world has prompted a desire by the public, governmental bodies, and professional organizations to create mechanisms that evaluate the utilization of ART. Advances in the arena of assisted reproductive technologies (ART) are accompanied by ethical and societal concerns. Legislation and professional societies have attempted to address these concerns for some time. For example, in 1986, the American Fertility Society first published guidelines for the ethical implementation of ART in the United States [7]. The dynamic nature of ART and the rapid evolution of the field result in constant paradigm shifts that require frequent and comprehensive evaluation by professional organizations and society alike.

In the 1980's, concerns surrounding ART focused on the safe administration of gonadotropins, transparency of pregnancy data from clinics, and addressing economic barriers to ART access. Some of these issues, such as reporting requirements for ART pregnancy results, have also been mandated with legislation in many nations [8]. Furthermore, ART reporting requirements generally include the number of embryos transferred. This measure has been extremely important in correlating the risk of multiple gestations with the transfer of 2 or more embryos. However, in many nations, reporting regulations are not accompanied by legislation defining practice patterns. For example, in the United States, while physicians are required to report the number of embryos transferred in an IVF cycle, there are no laws that state the allowed number of embryos transferred [8].

Through centralized mandatory reporting registries, general estimates of IVF activity are available in many nations. In an effort to define current IVF statistics and to make this information more transparent and available to patients, the Fertility Clinic Success Rate and Certification Act of 1992 was created in the United States [8]. This law requires clinics providing IVF in the United States to report specific information regarding IVF cycles, including pregnancy rates [6]. This reporting data is only reported on IVF cycle outcomes and does not include detailed information regarding the maternal or paternal medical history [6]. In other countries, similar national registries exist [5], making it possible to evaluate data from IVF cycles on both a national and international scale. A detailed accounting for ART reporting and regulations across the globe is available from the International Federation of Fertility Societies (IFFS) [5]. In their 2010 report, the IFFS reported ART outcomes data from 59 countries [5].

Such laws were implemented in an attempt to ensure that patients may be informed as to which clinics have superior ART pregnancy results. In some instances, however, this has led to some clinics "cherry picking" patients to improve their overall pregnancy results. This has actually become a barrier to receiving ART for many patients with a relatively poor pregnancy prognosis.

4. Practice Regulations and Multiple Gestation Pregnancies

Federally mandated regulations, however, are not limited to registries. Increasingly, nations have enacted legislation that defines the parameters for acceptable practice of ART. The transfer of multiple embryos in a single cycle increases the rates of multiple births [9]. Because of the increased social costs and health risks associated with multiple births, legislation or guidelines from professional societies have been introduced in many countries restricting the number of embryos that may be transferred per IVF cycle in an effort to limit the incidence of multiple gestations [9–11]. Indeed, a study in the United Kingdom found that the total health care system costs following a singleton birth were £3313, £9122 following a twin birth and £32,354 following a triplet birth [9]. Additionally, the health risks, both to the mother and the infant, increase dramatically with increasing number of infants [9]. In the United States in 2007, the number of embryos transferred per cycle ranged from 2.2 in women under 35 to 3.1 in women over 40 years of age (CDC). Multiple birth rates in the United States in 2007 ranged from approximately 35% in women under 35 to 15% in women over the age of 40 [12]. In Europe, the approximate number of embryos transferred in the year 2006 was one (22%), two (57%), three (19%), or four (1.6%) [13]. In 2007, 79.2% of European births were singletons, with a twin rate of 19.9% and a triplet rate of 0.9% [5].

Pregnancy rates associated with IVF are high compared to those seen in the early days of the procedure. The current efficiency of IVF is more cost effective and efficacious in achieving pregnancy than other modalities, such as injectable gonadotropins coupled with intra uterine insemination (IUI), which traditionally some have preferred [14]. The increased efficiency of IVF has also resulted in an increased rate of multiple gestations. Recent data suggests that single embryo transfer, coupled with subsequent frozen embryo transfer, results in equivalent pregnancy rates compared with the transfer of multiple embryos, without an increase in multiple pregnancy rates [11]. Additionally, single embryo transfer would inherently decrease maternal and infant health risks associated with multiple gestation pregnancies [9]. Therefore, a trend toward single embryo transfer is likely to increase in the future.

Variability of legislation regulating IVF exists in different countries and even states/provinces within a single nation [6]. For example, in an effort to minimize multiple gestation pregnancies resulting from ART, some laws place limits on the number of embryos that may be transferred, cryopreserved, or fertilized per IVF cycle [5, 6, 15, 16]. In some cases, these regulations or fiscal pressures result in couples traveling across international border to obtain treatments that are unavailable in their native country [17]. This practice, known as cross-border reproductive care (CBRC), is thought to account for as much as 10% of the total IVF cycles performed worldwide [17, 18].

5. Financial Aspect for IVF Treatment

Perhaps one of the most obvious ethical challenges surrounding ART is the inequitable distribution of access to care. The fact that significant economic barriers to IVF exist in many countries results in the preferential availability of these technologies to couples in a position of financial strength [19]. The cost of performing ART per live birth varies among countries [4]. The average cost per IVF cycle in the United States is USD 9,266 [20]. However, the cost per live birth for autologous ART treatment cycles in the United States, Canada, and the United Kingdom ranged from approximately USD 33,000 to 41,000 compared to USD 24,000 to 25,000 in Scandinavia, Japan, and Australia [14]. The total ART treatment costs as a percentage of total healthcare expenditures in 2003 were 0.06% in the United States, 0.09% in Japan, and 0.25% in Australia [4]. Some have maintained that the cost for these cycles pales in comparison to the social advantages yielded by the addition of productive members of society [21]. This is especially true in societies that have a negative or flat population growth rate coupled with an aging population [21].

The funding structure for IVF/ART is highly variable among different nations. For example, no federal government reimbursement exists for IVF in the United States, although certain states have insurance mandates for ART [4, 19, 22]. Many other countries provide full or partial coverage through governmental insurance [4, 9]. In many instances, long waiting times for IVF through these government programs encourage couples to seek treatment in private fertility centers that accept remuneration directly from the patients [4, 23, 24]. In the United Kingdom, for example, only approximately 25% of all IVF cycles performed are funded by the National Health Service [9].

6. Preimplantation Genetic Testing

Preimplantation genetic screening (PGS) and diagnosis (PGD) offer the unique ability to characterize the genetic composition of embryos prior to embryo transfer. Given the recent successes of these technologies, the broader implementation of this technology in the future is likely. Although controversial, using PGD to choose embryos solely on the basis of gender is currently being practiced [25, 26]. Sex selection in the proper setting may offer a substantial health benefit. For example, choosing to transfer only embryos of a certain sex may confer a therapeutic benefit if used to avoid a known sex linked disorder. However, sex selection PGD purely for the preference of the parents could conceivably, if practiced on a large scale, skew the gender proportions in certain nations where one gender is culturally preferred.

In the near future, with refinements in microarray technology and the defining of genetic sequences associated with certain physical characteristics, it is conceivable that specific physical or mental characteristics may be evaluated to guide the decision as to which embryos to transfer. This possibility raises concerns on both ethical and practical levels. Of more concern is the possibility that in the future, technology will permit the manipulation of genetic material within an embryo. Rigorous public and scientific oversight of these technologies is vital to ensure that scientific advances are tempered with the best interests of society in mind.

7. Fertility Preservation

Female fertility is well documented to decrease with age [27, 28]. Consequently, much research has been conducted aimed at preserving female fertility before advanced age is realized. Additionally, fertility preservation for individuals afflicted with cancer has important implications as often the chemotherapeutic agents used to treat cancer are toxic to the ovary and result in diminished ovarian reserve and reduced fertility. While techniques for freezing sperm and embryos are well established, techniques for freezing oocytes and ovarian tissue are still considered experimental [29]. Multiple techniques including oocyte cryopreservation and preservation of strips of ovarian cortex with subsequent reimplantation and stimulation have been described, with some pregnancy success [30–33]. Fertility preservation for cancer patients using *in vitro* maturation (IVM), oocyte vitrification and the freezing of intact human ovaries with their vascular pedicles have also been reported [34]. As of 2008, more than 5 babies had been delivered through IVF following ovarian tissue transplantation [35]. Many have suggested that, prior to being treated for cancer, women should be offered fertility preservation measures as outlined above [34].

Recently, several laboratories have demonstrated the ability to successfully cryopreserve oocytes following an IVF cycle. These developments have profound implications. As the birth control pill gave women the ability to prevent pregnancy, oocyte cryopreservation may give women the flexibility to preserve their fertility potential, starting at a young age, while postponing childbearing. However, as this technology at the present time in many countries is generally only available to those with financial means. This poses ethical and social issues that will certainly see more attention in the future.

8. Gamete Donation

The use of donor gametes, either in the form of donor sperm or donor oocytes, is commonplace in ART. The use of donor sperm can be traced to the 1800's [36]. In the mid 1980s, oocyte donation was introduced [36]. In recent years, issues surrounding the use of donor gametes have become increasingly visible [37]. Women donating oocytes must undergo IVF. Due to the inherent medical risks associated with IVF, including ovarian hyperstimulation syndrome and surgical risks, a central concern of allowing women to be oocyte donors includes adequate informed consent [37]. Consent, in addition to outlining these medical risks, should include counseling regarding the emotional benefits and risks of donation with an emphasis that long-term data regarding these risks are lacking [37]. Additionally, it is considered an ethical prerequisite that oocyte donors participate voluntarily and without coercion or undue influence [38]. Some have

expressed concern that financial compensation of oocyte donors may lead to exploitation as women may proceed with oocyte donation against their own best interests, given the inherent medical risks involved [39]. The concept of commodification, that any "buying or selling" of human gametes is inherently immoral, is an additional argument used against remunerating women serving as oocyte donors [39]. Due to the substantial controversy surrounding oocyte donation, especially the amount of financial compensation may be given to an oocyte donor, federal regulations governing this practice are constantly evolving and differ substantially from country to country [39].

Another ethical and legal issue surrounding the use of donated gametes is to what extent the anonymity of the donor should be preserved. The issue of anonymity as it relates to gamete and embryo donation is emotionally charged. Indeed, the ability of human beings to know their genetic roots is universally important, at the core of self identity. Either egg and sperm donors may choose to or not to be anonymous, though the vast majority in both groups generally chooses anonymity [40]. The American Society for Reproductive Medicine has identified four levels of gamete donor information sharing depending on the wishes of the donor and recipient parties [37]. Recently, however, there is, increasing consideration of the rights of offspring as it relates to donor gametes and anonymity [40]. Advocates for allowing either gamete donors or their offspring to break anonymity cite the medical advantages of sharing medical information with their genetic offspring, in the case of the donor, or learning about their genetic history directly, in the case of offspring [41, 42]. Others simply argue that both donors and offspring have an inherent right to meet and develop a relationship [43]. Recent court rulings suggest that these rights will become more visible in the future. For example, in the British case *Rose v Secretary of State for Health [2002] EWHC 1593*, the court ruled that based on the Human Rights Act, donor offspring could obtain information about their genetic parents despite previously established anonymity [43]. The ethical and legal issues surrounding anonymity and gamete donation are sure to be a centrally debated issues within the field of ART for the foreseeable future.

9. Embryo Donation

IVF cycles often result in couples transferring several embryos and cryopreserving other embryos produced by the cycle, presumptively for the purpose future pregnancy. However, in many instances, these surplus embryos are never used by the genetic parents and therefore are stored indefinitely [44]. The number of such embryos stored internationally is surprisingly high. In the United States alone, it is estimated that over 400,000 embryos are currently cryopreserved, many of which will not be used by their genetic parents [44]. The ethical and moral issues surrounding how to deal with these surplus embryos have been the source of much debate. In general, four possible fates for these embryos exist [44]:

(1) thawing and discarding,

(2) donating to research,

(3) indefinite storage,

(4) donating the embryos to another couple for the purposes of uterine transfer.

All of these strategies have staunch supporters and detractors. Not surprisingly, there are a myriad of laws in different countries governing many aspects of how a human embryo that has been cryopreserved may be handled [44, 45]. The use of embryos for the purpose of research, specifically as it relates to human stem cells, has also been a source of fierce debate internationally and has resulted in substantial regulation that varies substantially from nation to nation [46–49].

10. Surrogacy and Gestational Carriers

Another topic of ethical, social, and legal debate surrounds the use of surrogacy and gestational carriers. Surrogacy is defined as a woman who agrees to carry a pregnancy using her own oocytes but the sperm of another couple and relinquish the child to this couple upon delivery [50]. A gestational carrier, by contrast, involves a couple who undergoes IVF with their genetic gametes and then places the resultant embryo in another woman's uterus, the gestational carrier, who will carry the pregnancy and relinquish the child to this couple upon delivery [50]. Currently, the use of gestational carriers is far more common than that of surrogates [50].

As with donor gametes, surrogates and gestational carriers are subject to significant medical and emotional risks from carrying a pregnancy and undergoing a delivery [50]. As such, extensive counseling and meticulous informed consent are required [50]. Some also are concerned that the use of surrogates and gestational carriers is a form of "child selling" or the "sale of parental rights" [51]. Additionally, the rights of the surrogate or gestational carrier to not relinquish the infant following deliver are not well described [50]. In fact, legal precedent in some states within the United States has actually upheld the right of a birth mother, regardless of genetic relation to the child, to retain parental rights despite the existence of a preexisting gestational carrier contract [50].

Another central concern surrounding the use of surrogates and gestational carriers is the possibility that financial pressures could lead to exploitation and commoidification of the service [50–53]. The mean compensation for a gestational carrier in the United State in 2008 was estimated at approximately $20,000 [50]. In contrast, a gestational carrier in India receives an average of $4,000 for the same service [52]. Regulation of surrogates and gestational carriers varies widely from nation to nation and even within regions of individual countries [50, 52–56]. Due to these financial and legal considerations, international surrogacy has emerged as an emerging industry, especially in developing nations [52]. This practice has exacerbated the already difficult ethical and legal issues surrounding gestational carriers [52]. At the present time, issues surrounding issues of individual rights, commoidification, exploitation, citizenship of the offspring

of international gestational carriers, and even fair trade are largely unresolved internationally [52, 55].

11. Possible Deleterious Effects of ART

There are questions that remain outstanding regarding the use of IVF. Conflicting data exists about the risks of IVF on the developing embryo. Multiple studies have failed to find a clinically relevant association between IVF or embryo cryopreservation and adverse maternal or fetal effects [57–59]. Other studies have suggested that infants of IVF pregnancies may be at a small but statistically significant increased risk for rare epigenetic and other abnormalities [60–62].

Despite this controversy, there is a general consensus that IVF confers a small but measurable increased risk for a variety of congenital abnormalities including anatomic abnormalities and imprinting errors as compared to the general population [63]. Some maintain, however, that this is secondary to an increased baseline risk for these problems in the population of infertile patients [63]. Regardless of the cause, this small increased risk, while statistically significant with extremely large sample sizes, will likely not be a powerful enough factor to dissuade infertile couples from pursuing parenthood through IVF.

12. Conclusion

ART has emerged as one of the most widely adopted and successful medical technologies in the last century. While giving hope to millions of couples suffering from infertility, ART also has presented new ethical, legal, and social questions that society must address. Many countries have taken steps to regulate certain aspects of ART. Specifically, what regulations and laws should be in place for ART reporting, social inequities that may arise from financial barriers to ART, genetic testing, emerging laboratory techniques that have improved embryo and gamete survival when cryopreserved, and an individual's right to their genetic offspring in the setting of gamete or embryo donation are aspects of ART which will become increasingly controversial and debated into the future.

However, the lion's share of ethical and legal questions that exist surrounding ART have yet to be resolved. Society must reconcile how to fund ART in a responsible and equitable manner to increase access to care. Additionally, the myriad of unresolved issues surrounding gamete and embryo donation must be addressed in greater detail in future social and legal dialogues.

ART is a field that is dynamic and ever changing. In areas of ART such as preimplantation genetics, new technologies continually change the capabilities of ART. Due to the rapidly evolving nature of the ART, legislation is often unable to keep pace and address all of the ethical and legal issues that are constantly emerging in the field. It is therefore incumbent upon physicians to continually monitor these issues and ensure that ART technologies are offered and delivered in a manner that balances patient care with social and moral responsibility.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

Infertility and Adenomyosis

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Classically, the diagnosis of adenomyosis has only been possible on a hysterectomy specimen, usually in women in their late fourth and fifth decades, and, therefore, evaluating any relationship with infertility was simply not possible. As a consequence, to this day, no epidemiologic data exists linking adenomyosis to a state of subfertility. Today, new imaging techniques have enabled a noninvasive diagnosis at a much earlier time and a number of single-case or small series reports have appeared showing that medical, surgical, or combined treatment can restore fertility in women with adenomyosis, an indirect proof of an association. At the functional level, several anomalies found in the so-called junctional zone, or inner myometrium, in adenomyosis patients have been shown to be associated with poor reproductive performance, mainly through perturbed uterine peristalsis. Additional evidence for an association comes from experimental data: in baboons, adenomyosis is associated with lifelong primary infertility, as well as to endometriosis. Finally, indirect proof comes from studies of the eutopic and ectopic endometrium in women with adenomyosis proving the existence of an altered endometrial function and receptivity. In conclusion, sufficient indirect proof exists linking adenomyosis to infertility to warrant systematic clinical studies.

1. Introduction

Adenomyosis has been defined as the "benign invasion of endometrium into the myometrium, producing a diffusely enlarged uterus which microscopically exhibits ectopic non-neoplastic endometrial glands and stroma surrounded by a hypertrophic and hyperplastic myometrium" [1]. Two separate pathogenetic theories have been advanced to explain its formation: an origin from the invagination of the deepest portion of the endometrial mucosa between bundles of smooth muscle fibres of the myometrium, or along the intramyometrial lymphatic system; a metaplastic process initiating from ectopic intramyometrial endometrial tissue produced *de novo* [2].

It has long been suspected that the presence of adenomyosis provokes a condition of subfertility. Unfortunately, unlike endometriosis where an association with infertility has been all but proven [3], classically the diagnosis of adenomyosis has been, until recently, carried out on hysterectomy specimens and in women in their late thirties and forties. This reality made it impossible to evaluate its effects on fertility [4].

Nonetheless, as early as 1988, Honoré et al. [5] published three cases of "adenomyoma," a rare, localised form of adenomyosis [6], in young infertile women in whom surgery was carried out because of a diagnosis of leiomyoma. Based on this finding, they advocated early diagnosis and surgical intervention.

The situation changed some 25 years ago, with the identification through magnetic resonance (MR) imaging of a new functional uterine zone: the junction between the endometrium and the inner myometrium, named uterine junctional zone (JZ), measuring, in healthy young women, 5 mm in thickness or less [7]; this zone is clearly thickened in the presence of adenomyosis [8]. This was followed by attempts at identifying both the JZ and the presence of adenomyosis through ultrasonography [9], a technique now validated using a coronal section of the uterus obtained by three-dimensional trans vaginal sonography (TVS) [10].

Today both techniques can be utilised for an accurate evaluation and measurement of the JZ and of its alterations in the presence of adenomyosis, since they have good sensitivity and specificity. With regard to TVS, in a histologically controlled study Exacoustos et al. [10] have shown that the presence of myometrial cysts represents the most specific 2D-TVS feature for a correct diagnosis of adenomyosis, with a specificity of 98% and an accuracy of 78%. In their study, the most sensitive feature was the presence of a heterogeneous myometrium (sensitivity: 88%; accuracy: 75%). For 3D-TVS the best sensitivity is given by a JZ difference in thickness ≥4 mm and JZ infiltration and distortion (88%), with an accuracy of 85% and 82%, respectively. Exacoustos et al. concluded: "for 2D-TVS and 3D-TVS, respectively, the overall accuracy for diagnosis of adenomyosis was 83% and 89%, the sensitivity was 75% and 91%, the specificity was 90% and 88%, the positive predictive value was 86% and 85% and the negative predictive value was 82% and 92%."

According to Dueholm et al. [11], MR imaging is superior to TVS for the diagnosis of adenomyosis, having equal sensitivity but a higher specificity (sensitivity: MR 0.70 (0.46–0.87) and TVS 0.68 (0.44–0.86) ($P = .66$); specificity: MR 0.86 (0.76–0.93) and TVS 0.65 (0.50–0.77) ($P = .03$)). They point out that MR diagnostic accuracy improves when excluding uteri >400 mL and conclude that the combination of MRI and TVS produces the highest level of accuracy for exclusion of adenomyosis. In addition, measurement of the difference in junctional zone thickness may optimize the MR diagnosis. In the study by Dueholm et al., the combination of MRI and TVS was most sensitive (0.89 (0.64–0.98)), but produced the lowest specificity (0.60 (0.44–0.73)). Exclusion of uteri >400 mL from the analysis improved the diagnostic precision of MRI, but not that of TVS. The diagnostic accuracy at MRI was also improved by calculating the maximum difference between the thinnest and thickest junctional zone (JZdif) (i.e., > or = 5–7 mm).

The availability of noninvasive, imaging techniques enabling a preoperatory diagnosis of adenomyosis [12–15], have not only revolutionised treatment [16], they have renewed interest by the scientific community on an otherwise neglected condition, creating a flurry of research activities leading also to improved knowledge of the relationship between adenomyosis and endometriosis [17].

2. The Uterine Junctional Zone

The inner myometrium, or junctional zone myometrium, also called "archimetra" [18] possesses a specific characteristic that distinguishes it from other similar junctions in the human body: it lacks a recognisable protective layer or membrane, forcing endometrial glands into direct contact with the myometrium. MR, T2-weighted images of the uterus, display in healthy women of reproductive age three distinct layers [14]: (1) the endometrial mucosa or innermost stratum, providing a signal of high intensity; (2) the already mentioned, intermediate area immediately subendometrial, giving a signal of low intensity and named junctional zone myometrium; (3) an outer zone extending all the way to the serosal layer, or outer myometrium, with a medium-signal intensity.

Recently, a classification for adenomyosis has been proposed by Gordts et al. [6]: simple JZ hyperplasia (zone thickness ≥8 mm but <12 mm on T2-weighted images, in women aged 35 years or less); partial or diffuse adenomyosis (thickness ≥12 mm; high-signal intensity myometrial foci; involvement of the outer myometrium: <1/3, <2/3, > 2/3), adenomyoma (myometrial mass with indistinct margins of primarily low-signal intensity on all MR sequences). Unfortunately, this classification has never been debated or submitted to a consensus meeting and, therefore, remains to be validated.

Research carried out over the last two decades has now provided proper information on the nature and functions of the JZ. It has been shown that the zone undergoes cyclical changes in its thickness that mimic that of the endometrium and are characterised by maximum growth between days 8 and 16 [12], making it a hormone-dependent structure that governs uterine peristalsis outside pregnancy. During postmenopause, under suppression of ovarian activity with hormonal contraception, or following administration of gonadotropin releasing-hormone analogues (GnRH-A), the myometrial layers become indistinct on MR imaging, although use of hormone replacement therapy results in the reappearance of the typical zonal anatomy [19].

Transabdominal ultrasound imaging has now shown the presence in the myometrium of distinct contraction waves; this peristaltic activity originates exclusively from the junctional zone, while the outer myometrium remains quiescent. During the follicular and periovulatory phases, contraction waves have a cervicofundal orientation and their amplitude and frequency increase significantly towards the time of ovulation [20]. These waves are probably implicated in many aspects of the physiological reproductive process: endometrial differentiation [21], menstruation [22], sperm transport [23], and implantation [24]. Myometrial contractions have the ability to transport and preferentially direct microspheres placed in the vagina to mimic spermatozoa, towards the peritoneal opening of the tubes on the side of the dominant follicle [25]. During the luteal phase, uterine contractility decreases and myometrial contraction waves become short and asymmetrical, often running in opposing directions. This reduced activity may help the implantation process that, classically, takes place near the fundus and possibly facilitates local supply of nutrients and oxygen. In addition, in humans, interstitial and intravascular trophoblast invasion goes beyond the endometrium and involves the junctional zone, but not the outer myometrium [26]. Finally, 7 days after ovulation, at a time coinciding with embryo implantation there is a focal disruption of the junctional zone signal intensity [24].

Given the fact that the presence of adenomyosis involves alterations of the myometrium, as well as of the ZJ, a critical area for successful reproduction, it seems reasonable to hypothesise the existence of a relationship with subfertility [27]. Evidence is also available of a close relationship between the occurrence of adenomyosis and the structural and functional defects in the eutopic endometrium and the myometrial

uterine JZ. These abnormalities in turn may cause implantation failure and infertility [28].

3. Evidence Linking Adenomyosis to Infertility

As already stressed, the advent of high resolution imaging techniques has completely revolutionised our ability to identify the presence of milder forms of adenomyosis and, therefore, to explore a possible link with infertility.

Although no epidemiologic evidence exists, indirect data are available and provide a good case for an association between adenomyosis and infertility. Already fifteen years ago, de Souza et al. [27] reported an incidence of 54% myometrial JZ hyperplasia (a clear sign of adenomyosis) in subfertile patients complaining of menorrhagia or dysmenorrhoea. The mean age of these women was 34 years and some 70% of them were nulliparae. Several studies have confirmed the early work of de Souza: the disease can be present even in young women and be associated with both pelvic endometriosis and infertility and therefore may well represent a contributing factor [29–32]. This is more so since today, in western countries, an increasing number of women delay their first pregnancy until their late 30 s or early 40 s and, as a consequence, more women are found to have adenomyosis in fertility clinics during their diagnostic work-up [33].

Some evidence of an association can also be derived from reports of infertile women achieving pregnancy after being treated for adenomyosis. The first agents utilised for this purpose were GnRH-A [34] and several case reports or small series have been published with the analogue given alone, or in combination with surgery. In this connection, it has been found that, in IVF cycles, MR evaluation of junctional zone thickness is the best predictive factor of implantation failure [35], in the sense that an increase in JZ diameter is inversely correlated to the implantation rate. In fact, a thickened JZ is an independent factor for embryo implantation failure, and it is especially independent from embryo quality, infertility subtype, or patients age [36]. This observation has important clinical implications: in the presence of JZ thicker than 10 mm it becomes necessary to discuss with the patient whether to proceed immediately with IVF, or to postpone the procedure and carry out treatment with a GnRH analogue, a procedure that has the potential to reduce JZ thickness as assessed by successive MR [37]. Early results [38] seem to confirm an improvement of IVF results after this kind of therapy. In addition, prolonged pretreatment with GnRH-A before IVF has been reported to improve clinical pregnancy rates in infertile women with endometriosis [39]. Although, no data are available on women with adenomyosis, it seems reasonable to infer that also in this case pre-treatment may be beneficial.

Analogues can offer many advantages as a treatment for adenomyosis-associated infertility, over and above the hypooestrogenic state they produce: therapy with GnRH-A decreases expression of aromatase cytochrome P450 in the eutopic endometrium of women with adenomyosis and endometriosis [40] and it is well known that this enzyme is overexpressed in patients with these conditions. In women with adenomyosis, GnRH-A can suppress the generation of peroxynitrite, a compound known to cause tissue injury [41].

Several reports exist on the use of GnRH-A in the treatment of adenomyosis-associated infertility; the first case, ending in miscarriage, dates back to 1993 [42]; this was followed in 1994 by the first report of a successful term pregnancy [43]. Reports of small series of successful combined (GnRH-A plus surgery) treatment in women with adenomyosis seeking pregnancy have also appeared [44–47]. Additional evidence of a linkage between adenomyosis and infertility comes from a small Japanese study using an intrauterine system releasing danazol, in which three out of four infertile women conceived after removal [48]. A second, more recent option is offered by the levonorgestrel-releasing IUS, known as Mirena, although—so far—it has been only utilised for the relief of symptoms associated with adenomyosis [49, 50]. Finally, surgery has also been utilised to restore fertility in women with adenomyosis; a new conservative surgical technique called "adenomyomectomy" seems to offer good results (a pregnancy rate of around 50%) [51].

On a different front, there is good experimental evidence linking adenomyosis to infertility. Indeed, in baboons adenomyosis is not only strongly associated with lifelong primary infertility, but also statistically significantly associated to endometriosis [52]. Finally, it has been known for some times that a subfamily of homeobox genes named Abdominal B (*AbdB*), are involved in the developing urogenital system in vertebrates. Satokata et al. [53] have mutated one of the *AbdB* genes named *Hoxa10* in mice and observed that female homozygotes ovulate normally, but—if pregnancy occurs—in the great majority of animals it ends with the death of all embryos, and abortion occurs at the time when the *Hoxa10* gene should be expressed (2.5 to 3.5 days after coitus). This means that proper expression of the maternal *Hoxa10* gene is necessary to maintain viability of the preimplantation embryo, and, recently, it has been proven that in women with adenomyosis the expression of *Hoxa10* gene is decreased during the secretory phase of the cycle, a possible explanation for the observed lower implantation rate in women with adenomyosis [54].

4. Possible Mechanisms Involved in Adenomyosis-Associated Infertility

The above-mentioned data not only support the hypothesis that adenomyosis may be associated with infertility; they also provide a number of clues as to which mechanisms may be involved. Indeed, structural and functional defects of the uterine JZ, as well as the existence of several dysregulated proteins can cause implantation failure. In addition, a number of other conditions can, in theory at least, impair fertility: the presence of abnormal levels of intrauterine free radicals; an aberrant endometrial development throughout the menstrual cycle, possibly as a consequence of an abnormal local steroid metabolism; a lack of expression of some of the "implantation markers"; an altered function of genes essential for embryonic development.

4.1. Dysregulation of Myometrial Architecture and Function. An interesting comparative analysis of protein expression in adenomyotic tissue and in normal myometrium has been conducted by Liu et al. [55], who found that in women with adenomyosis there are 12 dysregulated protein spots and were able to identify 10 of them by mass spectrometry. In subjects with adenomyosis myocytes exhibit cellular hypertrophy, to the point that smooth muscle cells become ultrastructurally different from smooth muscle cells of normal uteri. The JZ shows cellular and nuclear hypertrophy, abnormal nuclear and mitochondrial shape, and a number of other abnormalities that may cause a disturbance in the normal calcium cycling in the affected myocytes, with a subsequent loss of normal rhythmic contractions [56]. Although it is too early to conclude that these phenomena may be implicated in creating a subfertility condition, it has been shown that adenomyosis causes an impairment of the rapid, sustained, and accurately directed sperm transport in the uterus consequent to the destruction of the normal architecture of the JZ myometrium [18]. These patients also show a reduced uterotubal transport capacity that progressively decreases with increasing severity of the disease; also, a major disruption of uterotubal transport has been detected using radionuclides in women with diffuse adenomyosis and primary infertility [57, 58]. Finally, adenomyosis is associated to a loss of nerve fibres at the endometrial-myometrial interface [59].

Although no definite explanation exists for the role of a thickened JZ in reducing implantation rates, the hypothesis has been brought forward that, under abnormal hormonal influence, ectopic endometrial glands can trigger an "inflammatory" reaction. This would be mediated by cytokines, prostaglandins, or other still unspecified factors and would determine smooth muscle proliferation that, in turn, would alter uterine contractions [60].

4.2. Altered Endometrial Function and Receptivity. Within the endometrium itself, the presence of abnormal levels of free radical concentration represents a possible cause for infertility in adenomyosis patients. This is because a disruption of the balance between reactive oxygen species and antioxidants produces oxidative stress and an excessive free radical environment. In turn, this can damage fertilized eggs and inhibit embryo development and pregnancy, and Noda et al. [61] have shown that low concentrations of free radicals are necessary to create an appropriate environment for early embryonic development. In the presence of abnormal levels of free radicals the embryo may be attacked by activated macrophages or T cells, or be exposed to an excess of nitric oxide, which may result in early miscarriage [62]. A number of investigations have focused on enzymes producing or eliminating free radicals: two of them are particularly interesting in this context: xanthine oxidase (XO) that produces superoxide and superoxide dismutase (SOD) that eliminates it, while simultaneously producing hydroxyl radicals, that, in turn, can be eliminated by glutathione peroxidase (GPx). It has been shown that in women with adenomyosis, nitric oxide synthase (NOS), XO, SOD, and catalase levels do not fluctuate and are over expressed [63, 64]; interestingly, as already mentioned, administration of GnRH-A suppresses the expression of both eNOS and iNOS and the formation of peroxynitrite in adenomyosis [40].

Altered oxidative stress equilibrium is not the only mechanism through which a uterine environment hostile to the developing embryo can be produced in women with adenomyosis. Another important abnormality that may lead to an impairment of implantation has now been identified: in women with adenomyosis there is an aberrant endometrial development throughout the proliferative phase, and this may lead to abnormalities of the secretory phase. This seems due to altered endometrial vascularisation, an increase in regulatory factors involved in the endometrial vascular proliferation and changes in endometrial molecular markers of inflammation [65, 66]. Indeed, in subjects with adenomyosis, in both eutopic and ectopic endometria there is a significantly greater activity of the vascular endothelial growth factor (VEGF) of microvessel density [67] and of the hypoxia-inducible factor-1alpha [68]. Furthermore, a series of anomalies have been found in the secretion of interleukins in both eutopic and ectopic endometria of subjects with adenomyosis, again leading to a disruption of early events related to implantation. These anomalies involve an improper secretion of interleukins-6 [69], -8 [65], and -10 [70]. In conclusion, in women with adenomyosis an abnormal inflammatory response seems to exist and impair nidation.

There is a third mechanism through which an altered endometrium can lead to implantation failure: an abnormal intraendometrium metabolism. Since in adenomyosis IL-6 is over expressed [69], this could lead to increased oestrogen receptor expression and, indeed, the expression of the different isoforms of oestrogen receptor alpha (ER-α) and beta (ER-β) and progesterone receptor A (PR-A) and B (PR-B) are differentially modulated in uteri with adenomyosis compared with controls [71]. In addition, in the endometrium of subjects with adenomyosis there is over expression of cytochrome P450 [72]; this phenomenon increases local oestrogen production [73], and it has been shown that an over expression of endometrial aromatase significantly lowers clinical pregnancy rates (with similar numbers of retrieved oocytes and replaced embryos with respect to controls) [74]. In these women there is also a defect in progesterone receptors and loss of their action [75]; this altered balance between oestrogen and progesterone results in the persistence of ER-α, given that downregulation of this receptor is one of the primary functions of progesterone. The overexpression of ER-α in midsecretory phase reduces the secretion of beta 3 integrins, negatively regulated by oestrogens, thereby altering uterine receptivity [76]. The observed reduction in PR expression may even explain the poor response to progestational agents in women with adenomyosis [77].

In adenomyotic foci, ER-α staining does not vary during the menstrual cycle in either glands or stroma; conversely, there are no cyclical changes in its expression in the innermost or outer myometrium. Furthermore, ER-β expression in the proliferative phase is statistically significantly higher in the *functionalis* portion of the glands compared with

controls. Expression is similarly higher in the *basalis*, the stroma, the JZ, and outer myometrium, compared with control tissue where expression is weak and shows no statistically significant variation with the phase of the cycle [71]. The higher ER-β expression in the myometrium of adenomyotic uteri might thus contribute to the presence of the classically described myometrial hyperplasia [55].

A fourth mechanism that can lead to implantation failure is a lack of expression of some of the molecules, labelled "implantation markers," that are expressed by the endometrium and are required for the successful interaction between embryo and endometrium. In 2006, Yen et al. [78] have reported that during the implantation window, some of these markers are decreased in the endometrium of women with adenomyosis, suggesting that this may be one of the molecular mechanisms associated with a decreased implantation rate.

In particular, it has been demonstrated that the so-called Leukemia Inhibitory Factor (LIF) is associated with endometrial receptivity and is lower in women with infertility compared with healthy controls [79]. It has also been shown that LIF expression is decreased in the endometrium in women with adenomyosis during midsecretory phase and, when these women have a history of infertility, they show significantly lower LIF levels in uterine flushing fluid, compared with fertile controls [80].

One of them, the α-4, β-3 integrin appears on the surface of epithelial cells of both embryo and endometrium and on maternal surfaces around cycle day 19 to 20 and continues to be expressed during pregnancy [81]. Although it is not known whether its expression is modified in women with adenomyosis, it has been shown that integrin is missing in a subset of women with unexplained infertility and endometriosis [82]. Information on this and several other proteins such as glycodelin, osteopontin, and vitronectin that are believed to mediate trophoblast-endometrial interactions during implantation and are downregulated in women with endometriosis [83, 84], is still lacking in the case of adenomyosis, but it can at least be speculated that a mechanism of this kind may also be involved.

A fifth important factor that may be involved in creating an impairment of implantation in women with adenomyosis is the already mentioned altered function of the HoxaA10 gene. As stated above, this gene is part of a homeobox-containing transcription factors essential for embryonic development and proper adult endometrial growth during the menstrual cycle [85] and in women with adenomyosis expression its is significantly lower during the midsecretory phase compared with fertile controls [54].

5. Conclusions

At present it is impossible to show conclusively that adenomyosis can lead to subfertility or infertility because no epidemiologic studies have ever been carried out. At the same time, it is hoped that the introduction of MR and, even more, that of the more readily available 3D-TVS will facilitate early diagnosis and help collecting missing data. Notwithstanding this unsatisfactory situation, a careful look at molecular pathophysiology of the disease and at preliminary clinical results with a number of new techniques [28] can already help clarifying the situation although, the final answers lie in the execution of controlled clinical investigations.

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Clinical Study

Clinical and Demographic Characteristics of Women with Intrauterine Adhesion in Abuja, Nigeria

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Objective. Infertility menstrual abnormalities continue to constitute a significant bulk of gynecological consultation in Africa. Both of these problems are sometimes traced to intrauterine adhesions which are preventable in the majority of cases. *Study Design*. A retrospective analysis of intrauterine adhesions at the National Hospital Abuja, Nigeria, was carried out, covering the period from 1st September 1999 to 1st September 2004. A total of 72 cases were analyzed. Statical analysis was done using X^2. *Results*. The incidence of intrauterine adhesions was 1.73% of new patients. Mean age ± SD was 29.97 ± 4.82 years. Patients who were Para 0 to 1 constituted 81.9% of the total. Intrauterine adhesions significantly ($P < 0.02$) occurred in nulliparae. The majority (68%) were educated only up to secondary level which was significant ($P < 0.05$). Menstrual abnormalities were present in 90.3%. The commonest predisposing factor identified was a history of dilatation and curettage or uterine evacuation. *Conclusion*. Intrauterine adhesions are associated with lower educational status and low parity. Increasing educational targets nationally, poverty alleviation, nationwide retraining in manual vacuum aspiration, and wider application of this technique are recommended.

1. Introduction

Intrauterine adhesions (IUA) are also known by the term "uterine synechiae," and Asherman's syndrome. They were first described by Fritsch in 1944, although Asherman increased awareness of the condition significantly [1, 2]. IUA presents clinically with menstrual abnormalities usually hypomenorrhoea, oligomenorrhoea, or secondary amenorrhea. Sometimes, it may present with cyclical abdominal pain or with recurrent abortions [3].

The condition is caused usually by trauma to the endometrium, with infection occasionally contributing to the pathogenesis. Pregnant or recently pregnant uteri are more susceptible [1], and therefore IUA is more common in areas with high incidences of unsafe abortion [1]. A study in Nigeria implicated induced abortion in 23% of cases [4]. Other implicated factors include postpartum curettage, Caesarean section, myomectomy pelvic inflammatory disease, and repair of ruptured uteri [5].

IUA can be diagnosed through various methods. Hysterosalpingography is the most commonly employed method. Other methods include hysteroscopy (with or without 3-dimensional features), ultrasonography, and magnetic imaging [6–9].

Universally, the incidence of IUA is increasing mainly from curettage in induced, incomplete, and missed abortions, as well as in postpartum haemorrhage and also genital tuberculosis [10–13]. The manual vacuum aspiration (MVA) is a less hazardous procedure, with regard to complications such as IUA, compared to traditional dilatation and curettage [1, 14]. Training courses on this technique have been held in major cities in Nigeria, including Abuja, the Federal Capital Territory.

Bearing in mind the triad of poverty, ignorance, and disease, and the vicious cycle thus generated, this paper aims to highlight the clinical and demographic characteristics of women presenting with IUA at the new tertiary health facility in the Federal Capital Territory of Nigeria.

2. Materials and Methods

The case folders of patients with IUA who attended the gynaecological out-patients clinic of the National Hospital Abuja during a 5-year period between 1st September 1999

(the hospital inception date) and 1st September 2004 were retrieved from the medical records department. This hospital is the major specialist hospital serving the Federal Capital Territory of Nigeria.

Diagnosis was made following a hysterosalpingogram (HSG) and a negative progesterone challenge test. In some cases, ultrasound examination aided the diagnosis but confirmation was usually with HSG. Strict anatomical criteria of IUA were used and so cased with diagnosis of cervical synechiae were excluded from the study.

The age, parity, and educational status of the respondents were analyzed. Also the clinical presentation and any predisposing factors were analyzed. Level of significance was set at $P < 0.05$ (95% confidence interval). In 9 cases, there was more than 1 predisposing factor. In such cases, the most recent antecedent event was selected for the purpose of the study.

3. Results

During the period under study, there were 72 recorded cases of IUA out of a total of 4,165 new attendances at the gynaecological out-patients clinic. This gives a rate of 1.73%. All case-folders were retrieved and analysis was based on this figured as the denominator. The duration of symptoms ranged from 4 months to 7 years.

4. Age and Parity

The mean age of patients with IUA was 29.97 ± 4.82 years, with a range of 20–43 years. In terms of parity, 81.9% were of low parity (Para 0 or Para 1). These are show in Tables 1 and 2. IUA significantly occurred in nulliparae ($P < 0.02$, $X^1 = 5.6$, 1 df).

5. Educational Status

The majority (65.3%) were educated up to secondary level, as opposed to those with postsecondary education, and this was statistically significant ($P < 0.05$, $X^2 = 7.9$, 1 df). Two patients had primary level education. This is shown in Table 3.

6. Clinical Presentation

Table 4 shows the clinical presentation of the patients. Menstrual abnormalities were present in 90.3% of cases.

7. Predisposing Factors

The predisposing factors are show in Table 5. The highest contribution factor was a history of dilatation and curettage or uterine evacuation, either for a spontaneous or induced abortion or postpartum haemorrhage. Next in ranking order was a history of myomectomy, Caesarean section, manual removal of retained placenta, and pelvic inflammatory disease. No predisposing factors were found in one case record.

Table 1: Age distribution of patients with IUA.

Age	No. of patients	Percentage
20–24	5	6.9
25–29	21	29.2
30–34	37	51.4
35–39	8	11.1
40–44	1	1.4
Total	72	100

Table 2: Parity distribution of patients with IUA.

Parity	No. of patients	Percentage
0	43	59.7
1	16	22.2
2	7	9.7
3	3	4.2
4	3	4.2
Total	72	100

Table 3: Educational status of 72 cases of IUA.

Educational level attained	No. of patients	Percentage
Primary	2	2.8
Secondary	47	65.3
Tertiary	23	31.9
Total	72	100

8. Discussion

Comparison to state increase or decrease in prevalence is hindered by paucity of reports on the prevalence of IUA in the Federal Capital Territory of Nigeria. However, with reference to the study in Lagos, Nigeria [1], there is a lower prevalence of the condition in this region of the country. This may reflect the impact of the nationwide training in manual vacuum aspiration (MVA) techniques which began in 1989.

There were no patients in the under-20 age bracket while ages above 40 witnessed low numbers of patients. The majority (80.6%) were in the 25–35 age brackets, which is a reflection of the age pattern of reproductive activity. The mean age is similar to the study in Lagos, Nigeria [1].

As in Lagos study, the majority (18.9%) had a low parity of Para 0-1, and in this study, IUA significantly occurred in nulliparae ($P < 0.02$), illustrating the low reproductive potential and emphasizing the association of IUA with infertility [15]. Majority (65.3%) of patients with IUA, significantly had education only up to the secondary school level, which was more than double the number of those with tertiary education. The association of increased incidence of IUA with lower educational status is multifactorial. Lower educational status is more likely to lead to ignorance of appropriate health-seeking behavior or options when these teenagers or women are faced with health challenges. From a prevention or primary health care point of view, contraceptive usage or uptake is likely to be lower in this group, and this may then result in increased incidence of

Table 4: Clinical presentation.

Presentation	No. of patients	Percentage
Secondary amenorrhoea	30	41.7
Hypomenorrhoea	27	37.5
Oligomenorrhoea	8	11.1
Cyclical lower abdominal pain	4	5.5
Recurrent abortions	2	2.8
Normal menstruation	1	1.4
Total	72	100

Table 5: Predisposing factors in 72 cases of IUA.

Predisposing factors	Number	Percentage
Dilatation and curettage/uterine		
Evacuation	57	79.2
Myomectomy	6	8.3
Caesarean section	5	6.9
Manual removal of placentae	2	2.8
Pelvic inflammatory disease	1	1.4
Unexplained	1	1.4
Total	72	100

unwanted pregnancies. Taking into account the organization of health services and prevailing laws on induced abortions in Africa and other developing countries, this is likely to lead to procurement of backstreet and unsafe abortions, with resultant infection, trauma in this category of patients, and hence an increased incidence of intrauterine adhesions. Another reason could be that since lower educational status correlates with lower socioeconomic status in most developing countries, economic empowerment and ability to seek the appropriate healthcare in clinical scenarios such as irregular menstruation or infertility would be hindered [1, 4]. Thus such teenagers, adolescents, or women may patronize quacks or alternative medical practitioners who may then inflict harm on them via upper genital tract trauma or unnecessary dilatation and curettage. Even when this group of patients seek health attention from qualified medical practitioners, lower educational status is likely to hinder differentiation between a general practitioner and a qualified specialist gynaecologist. Again, those with lower educational status are less likely to comprehend the reasons for and implications of inadequate dosaging of prescribed antibiotics for pelvic infections, either in number, dose, or duration. Also the higher the educational status, the more likely that the teenagers, adolescents, or women will obtain their antibiotic and other medication from standard outlets such as pharmacies, taking into cognisance the prevalence of substandard and fake medication in this region of the world.

Most of the patients (90.3%) had abnormal menstruation as Ogedengbe et al. found in Lagos. Therefore the index of suspicion for IUA should be high in women of reproductive age with menstrual abnormalities. One patient had normal menstruation and the case was only discovered during the course of investigations for infertility.

Dilatation and curettage was the most significant predisposing factor identified, being present in about 79% of patients with IUA. This figure is higher than that obtained in Lagos, although the data here also includes uterine evacuation for postpartum haemorrhage. This emphasizes that this procedure should be carried out only by qualified medical practitioners properly trained in this regard. Curettage is advocated to be performed through MVA techniques employing blunt instrument techniques, rather than the traditional dilatation and curettage with sharp instruments [1]. The rationale is less trauma and injury to the endometrium in trained hands, the opposite of which is a crucial factor in the pathogenesis of IUA.

9. Conclusion

Intrauterine adhesions though relatively low in incidence at the gynaecological outpatients' clinic at the NHA, represent a preventable cause of infertility, which constitutes a significant bulk of a gynaecologist's workload in Africa. It is associated with lower educational status and low parity. This may be the case in other developing countries, particularly in Africa.

Increasing educational targets nationally, poverty alleviation, and nationwide retraining in MVA techniques are likely to reduce the incidence of the condition.

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Review Article

Sperm DNA Integrity Assessment: A New Tool in Diagnosis and Treatment of Fertility

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Infertility affects 15% of all couples. Although male infertility factors with reduced semen quality are contributing to about half of all involuntary childlessness, the value of standard semen parameters in prediction of fertility *in vivo* and choice of proper method for assisted reproduction is limited. In the search for better markers of male fertility, during the last 10 years, assessment of sperm DNA integrity has emerged as a strong new biomarker of semen quality that may have the potential to discriminate between infertile and fertile men. Sperm DNA Fragmentation Index (DFI) as assessed by the flow cytometric Sperm Chromatin Structure Assay (SCSA) can be used for evaluation of sperm chromatin integrity. The biological background for abnormal DFI is not completely known, but clinical data show that DFI above 30% is associated with very low chance for achieving pregnancy in natural way or by insemination, but not *in vitro*. Already when the DFI is above 20%, the chance of natural pregnancy may be reduced, despite other sperm parameters being normal. Thus this method may explain a significant proportion of cases of unexplained infertility and can be beneficial in counselling involuntary childless couples need of *in vitro* fertilisation.

1. Introduction

In western countries up to one-forth of couples in reproductive age are seeking medical help for involuntary childlessness [1]. Despite the significant developments in the area of fertility seen during the last decades about one-third of these couples will be undiagnosed without any explanation to their problems.

Although, the traditional semen parameters concentration, motility, and morphology are a golden standard in diagnosing of male infertility it has become apparent that none of these parameters recommended by the Word Health Organization (WHO) [2] are sufficient for the prediction of male fertility capacity. As the WHO parameters only address few aspects of sperm quality and function the discriminative power in relation to fertility is quite low [3, 4].

As a result, there has for long been searched for better markers of male fertility.

During the last decades the use of assisted reproductive techniques (ARTs) has increased substantially [1, 5]. In particular intracytoplasmic sperm injection (ICSI) is used to an increasing degree. While in the beginning of the era of ICSI the indication for treatment was severe male factor infertility, now also couples with normal sperm quality request are treated with ICSI. However, IVF and ICSI are symptomatic treatments where only 25–30% of the treatments result in a delivery [6]. One explanation to this limited success can be the lack of markers to find the underlying causes to subfertility and also a lack of methods to identify the type of ART treatment providing the most optimal chances of pregnancy in a given couple.

During the last decade the search for better predictors of male fertility has resulted in an increased focus on the sperm DNA integrity [7, 8]. Now number of sperm chromatin integrity assays is available. Among the most frequently used are the Comet assay (single cell gel electrophoresis) [9], the TUNEL (terminal deoxynucleotidyl transferase-mediated dUDP nick end labelling) assay [10], the sperm chromatin dispersion (SCD) [11, 12], and the sperm chromatin structure assay (SCSA) [13, 14]. The clinical value of these different tests varies; however, SCSA, first described by Evenson et al. [13] is shown to be an independent marker of fertility *in vivo* and may also help in selection of the most effective ART treatment in each individual couple [15]. This paper

will discuss how sperm DNA integrity assessment by help of SCSA can be used as a tool in diagnosis and treatment of infertility.

2. Aetiology and Diagnosis of Infertility

Involuntary childlessness, infertility can be a result of female, male as well as combined factors. It is a complex condition where often a mix of factors plays a role. In 20% of cases, the predominant cause is solely male related and, in another 30%, anomalies in both partners contribute to the childlessness [2]. Traditionally genital infections, endocrine disturbances, and immunological factors have been regarded as the most common causes of male subfertility. However, now more often genetic/molecular causes are identified as contributing factors [16], as,for instance, chromatin defects assessed as breaks in sperm DNA [7, 14, 17–19]. However, it is a fact that in 60–75% of the male-caused cases the aetiology of reduced semen quality remains unexplained and is therefore diagnosed as idiopathic infertility [2].

The golden standard in the diagnosis of male infertility or subfertility is an analysis of sperm concentration, motility, and morphology where WHO has set threshold levels for normality in regard to fertility [2]. However, this WHO sperm analysis is mainly performed by light microscopy of 100–200 spermatozoa and thus the analysis is biased because of a high level of subjectivity resulting in a high grade of intra- and interlaboratory variation [20, 21]. As a consequence a low predictive value of the WHO analysis is reported. Another drawback with the WHO parameters is that they only address few aspects of sperm quality and function. During the last decades, several other sperm function tests have been suggested to be used, including vital staining, hemizona assay, biochemical analysis of semen, antisperm antibody test, hypoosmotic swelling test, sperm penetration assay, reactive oxygen species (ROS) tests, and computer-assisted sperm analysis (CASA) [18]. However, as none of them have provided stable threshold values few are actually used in clinical routine [22].

Now an increasing amount of data demonstrate an association between sperm DNA damage and fertility [7, 14, 17, 19, 23–26]. It has been proposed that sperm DNA integrity could be a fertility predictor to be used as a supplement to the traditional sperm parameters [14, 15, 27].

3. Causes to Sperm DNA Damage

Spermatogenesis is a complex process [28] where damage of sperm chromatin structure can occur at any step (reviewed in [7]). DNA damage in sperm can be due to unrepaired DNA breaks during the spermatogenetic chromatin remodeling and packaging or abortive apoptosis during spermatogenesis. Among other suggested causes are the effect of endogenous endonucleases and caspases, exposure to a variety of genotoxic agents because of therapeutic reasons or because of occupational or environmental exposures, and finally, the action of oxidative sperm DNA damage [19]. Most likely often these factors are interrelating. Problems in the crossing-over process during spermatogenesis or deficiencies in the protamination process will likely make sperm more vulnerable to oxidative stress at a later occasion.

3.1. Remodeling and Packaging Problems. Meiotic crossing-over during spermatogenesis is associated with the programmed introduction of DNA double-strand breaks, expected to be ligated until the end of meiosis I [29]. Stage-specific introduction of transient DNA strand breaks during spermiogenesis has been described [30, 31]. DNA breaks are needed for transient relief of torsional stress, favouring the histone replacement with protamines during the final maturation form round to elongated spermatozoa [31, 32]. However, if and only if these physiological, normal temporary breaks are not repaired, DNA fragmentation in ejaculated spermatozoa or genetic mutations may occur [33].

3.2. Abortive Apoptosis. Another suggested aetiology of DNA damage is that breaks can arise through abortive apoptosis. Apoptosis of testicular germ cells occurs normally throughout life, controlling overproliferation [34]; however, it has been suggested an early apoptotic pathway, initiated in spermatogonia and spermatocytes, mediated by the Fas protein [35]. Sertoli cells in the testis express Fas ligand, which by binding to Fas leads to cell death through apoptosis [35].

3.3. Oxidative Stress. Oxidative stress (OS) caused by an imbalance between the antioxidant ability in seminal plasma and the production of reactive oxygen species (ROS) leading to the formation of oxidative products such as 8OHdG is the mechanism that probably most often lies behind sperm DNA defects. The sperm cell membrane is easily attacked by ROS with further detrimental effects on nuclear membranes as well as on sperm DNA [33]. Moreover, sperm lack antioxidants and DNA repair systems [36] and therefore protection of the offspring from the negative effects of male-induced DNA strand breaks is always dependant on the repair capacity of the oocyte and the early embryo. The main sources of ROS in semen are leukocytes and abnormal/dead spermatozoa in the semen [19, 37, 38]; however, increased scrotal temperature due to illness with fever [39–41], varicocele [42], increased age [43–47], and smoking [48–54] are also reported as sources.

4. Sperm DNA Integrity Assessment

Currently, four major tests of sperm DNA fragmentation are most frequently used, including the Comet assay (single cell gel electrophoresis) [9], the TUNEL (terminal deoxynucleotidyl transferase-mediated dUDP nick end labelling) assay [10], the sperm chromatin dispersion (SCD) test [11, 12], and the sperm chromatin structure assay (SCSA) [13, 14]. They all label single- or double-stranded DNA breaks; however unfortunately, most of the available techniques for detection of sperm DNA damage provide limited information on the nature of the DNA lesions detected and none of them enables us to depict the exact aetiology and pathogenesis of impairment of sperm DNA.

Comet assay is a fluorescence microscopic test, and TUNEL assay can be applied in both bright field/fluorescence microscopy and by flow cytometry. In Comet assay sperm cells are mixed with melted agarose and then placed on a glass slide. The cells are lysed and then subjected to horizontal electrophoresis. DNA is visualized with the help of a DNA-specific fluorescent dye and DNA damage is quantified by measuring the displacement between the genetic material of the nucleus comet head and the resulting tail. In the TUNEL assay, terminal deoxynucleotidyl transferase (TdT) incorporates labelled (by and large fluorescent) nucleotides to 3′-OH at single- and double-strand DNA breaks to create a signal, which increases with the number of DNA breaks. The fluorescence intensity of each analyzed sperm is determined as a "positive" or "negative" for sperm on a microscope slide. In a flow cytometer the fraction of positive sperm is represented by the cells above a threshold channel value on a relative fluorescent intensity scale.

SCSA is a flowcytometric test where sperm DNA breaks are evaluated indirectly through the DNA denaturability. The assay measures the susceptibility of sperm DNA to acid-induced DNA denaturation in situ, followed by staining with the fluorescence dye acridine orange [13, 14, 55]. By using a flow cytometer 5.000–10.000 sperm can be analyzed within few seconds and thus provide a less subjective result compared to the WHO analysis where only 1–300 cells are analyzed. Through a specific SCSA-software (SCSA-Soft) a scatter plot is created, showing the ratio of green and red sperm. The percentage of red sperm is called DNA fragmentation index (DFI) [14]. The sperm with the most intensive green colour is called high DNA stainable (HDS) sperm. It is still unclear precisely which mechanisms and types of DNA damage are lying behind DFI and HDS; however, it is believed that whilst DFI is related to the percentage of sperm with DNA breaks or protamine defects HDS is thought to represent immature spermatozoa [14].

So far, the SCSA is the only sperm DNA integrity assessment method which has demonstrated clear and clinically useful cut-off levels for calculating male fertility potential [13, 15, 27, 55]. The SCSA is a standardized test performed according to a strict protocol [14]. Apart from being subject to a very limited intralaboratory variation [56], however, the SCSA analysis has shown to be very robust to variation between laboratories. In an external quality control based on >180 samples, a high ($r = 0.8$) correlation was found between the values obtained by our laboratory and those from a control laboratory. Furthermore, the absolute DFI values obtained at two different laboratories, using different equipment, did not on average differ by >1% [57].

The advantage of SCSA is the objectivity of the test as well as the high reproducibility when running after the standardised protocol. Moreover, the clear cut-off levels in relation to fertility are maybe the most obvious benefit compared to other sperm DNA integrity tests. A disadvantage is that an expensive flow cytometer is required to run the analysis. Moreover, the test irreversibly damages spermatozoa; after analysis they cannot be used for fertilisation purposes.

Studies have demonstrated that these four sperm DNA integrity tests, the SCSA, TUNEL, Comet, and SCD assays, generally correlate moderately with each other (a coefficient of correlation between 0.4 and 0.7), which indicates that the tests likely are expressing different aspects of sperm DNA damage.

5. SCSA and the Chance of Pregnancy

Two population-based studies, including 165 and 215 couples respectively, [13, 55], have demonstrated that DFI as measured with SCSA is an excellent predictor of subfecundity in the normal population. In the interval of DFI 0–20%, the chance of spontaneous pregnancy was constant. When DFI was above 20% the chance of obtaining a spontaneous pregnancy was decreased and close to zero when the DFI level passed 30–40%. Even though DFI was below 20%, only 13% of all cycles resulted in a pregnancy. Therefore, in a normal population, not selected because of infertility problems, SCSA is a valuable tool to identify men who are at risk of not giving rise to a pregnancy. The same information is not possible to get from the traditional WHO sperm parameters. Even among men with low sperm concentration, poor motility or morphology there will be men with a certain potential of fertility [3].

In a case-control study of 137 infertile and 127 fertile men the risk of being infertile was found to be increased when DFI as measured by SCSA was above 20% in men with normal standard semen parameters, an odds ratio (OR) of 5.1, (CI: 1.2–23), compared to fertile controls [27]. If one of the WHO parameters was abnormal, the OR for infertility was increased already at DFI above 10% (OR 16, CI: 4.2–60). A DFI above 20% was found in 40% of the men with otherwise normal standard parameters. DFI was also shown to be an independent predictor of spontaneous pregnancy. Erenpreiss and coworkers found that 20% of the men with otherwise normal WHO sperm parameters had an SCSA-DFI above 20% [58].

The association between sperm DNA damage and the traditional semen parameters is shown to be only weak to moderate [57, 59]. It is also shown that 25–40% of infertile men may have normal standard sperm characteristics according to WHO criteria, but a DFI above 20–30% [15, 27, 58].

ART includes all technologies that involve the handling of sperm outside the body, as in intrauterine insemination (IUI), or handling of oocytes, sperm, and embryos as in *in vitro* fertilization (IVF) and ICSI [60]. In IVF, the spermatozoas ability to penetrate the zona pellucida of the oocyte is utilized. In ICSI, however, one single spermatozoon is selected and injected directly into the cytoplasm of the oocyte. Despite these obvious differences most studies report results from the three types of treatments together. In studies reporting the three treatment types separately, the number of patients included and thus the statistical power have been relatively low. The first SCSA study to indicate an association between sperm DNA damage and reduced pregnancy chances was published by Saleh et al. [61] who performed a small study where 12 of 19 couples had a DFI value as measured by SCSA above 28% and no pregnancy

was obtained. Also, Boe-Hansen and coworkers in a study on 48 IUI couples found only two couples with a DFI value above 30%, and neither here pregnancy was obtained [62]. Recently, in a study of 387 IUI cycles, we have shown that even in IUI SCSA-DFI can be used as an independent predictor of fertility [15]. Whilst the proportion of children born per cycle was 19.0% when the DFI value was below 30%, those with a DFI value above 30% only had a take-home-baby rate of 1.5%. In this group the OR for delivery in relation to DFI <30% was 0.07 (95% confidence interval (CI): 0.01–0.48). In the same study, 388 IVF and 223 ICSI cycles were included but it was not possible to find any threshold value for DFI that could predict the result of the treatments. However, surprisingly when DFI exceeded 30%, the result of ICSI was significantly better than IVF (OR for delivery was 2.17 (CI 1.04–4.51)). These data are in agreement with other previous smaller reports using TUNEL or COMET assays, showing that sperm DNA damage is more predictive in IVF and much less so in ICSI [63, 64].

Although fertilization and embryo development may be independent of sperm DNA integrity, it has been suggested that the postfertilization development of the pre-embryo can be impaired by such incomplete or aberrant sperm DNA repair by the oocyte leading to early miscarriages (reviewed in [65]) or in the worst cases diseases in the offspring [8, 66, 67]. In our study of about 1000 couples no relationship was seen between sperm DNA fragmentation and unexplained pregnancy loss [15].

The other SCSA parameter, HDS, was neither in ours nor in other studies found to be of predictive value of pregnancy.

6. Clinical Recommendations

SCSA represents a valuable tool in diagnosis and treatment of infertility. The usefulness of the method is first of all relating to *in vivo* fertility (spontaneous pregnancy and IUI) and in particular in the many couples diagnosed with unexplained infertility. In 20% of men presenting with otherwise normal semen parameters, the SCSA-DFI is above 30% and in these couples the chance of pregnancy is close to zero [15] that is why they should be directly referred to IVF/ICSI. Through such a strategy change one-fifth of all couples normally referred to IUI can avoid the huge burden an unsuccessful ART treatment represents.

In as many as 40% of all unexplained infertile couples the explanation may be related to a high DFI. In a couple having a DFI between 20 and 30% time to pregnancy will be longer than in a couple with a DFI level below 20%. This is important information that should be utilized in counselling the couple. Combining SCSA-DFI with assessment of traditional WHO sperm parameters is shown to give a higher precision in the prediction of fertility. If one of the WHO sperm parameters is that abnormal fertility becomes reduced already when DFI exceeds the level of 10%, whereas the DFI level is above 30%, couple should be revised directly to IVF/ICSI. However, the status is that still very few clinics have implemented sperm chromatin integrity testing and therefore most couples seeking help for infertility problems are not aware of their actual potential sperm DNA defects. One reason is the necessity of expensive equipment as, for instance, a flow cytometer as well as time, cost, and expertise to perform the analysis. On the other hand, sperm samples can be shipped to and analyzed on a centralized SCSA laboratory.

Sperm DNA breaks are mainly thought to be a result of oxidative stress. Some reports demonstrating a positive effect of antioxidant therapy in men with a high DFI have been published; however, the study populations have been small and data conflicting [45, 68–73]. In the future SCSA may also have the potential to give indications for a causal treatment of disturbances of male fertility.

Development of new improved tests depicting the cause of sperm DNA damage should be the next step in using sperm DNA integrity testing as a tool in diagnosis and treatment of infertility.

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Clinical Study

Cornual Polyps of the Fallopian Tube Are Associated with Endometriosis and Anovulation

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Background. The relationship between tubal cornual polyps and endometriosis and ovulatory disorders in infertile women is unclear. Our objective was to determine such an association from our database and review the literature. *Methods*. Twenty-two infertile women with tubal cornual polyps were assessed for coexistence of oligoovulation/anovulation and endometriosis with stratification for polyp diameter (large: ≥5 mm diameter, small <5 mm diameter). *Result(s)*. Oligoovulation/anovulation was more prevalent in women with large versus small tubal cornual polyps ($P = 0.0048$). Endometriosis was associated with both large and small polyps. *Conclusion(s)*. This case series confirms the association of tubal cornual polyps with oligoovulation/anovulation and endometriosis in infertile women. This case series is limited by a lack of controls.

1. Introduction

Tubal polyps, also known as tubocornual or cornual polyps, have been reported sporadically in the gynecologic literature. A common estimate of the prevalence of cornual polyps is 2-3% of the general population who undergo hysterosalpingography (HSG) (Figure 1) [1, 2]. However, the actual reported prevalence varies widely from 1.2% to 33% [3, 4]. Fernstroem and Lagerloef estimated a 10% prevalence of cornual polyps in all women, extrapolated from histological studies of posthysterectomy specimens [3]. It is believed that many polyps are undiagnosed, especially if diminutive in size, due to inaccurate interpretation of HSG imaging and/or an unfamiliarity of the examiner with this condition [2, 3].

Historically, cornual polyps were first described by Philipp and Huber in 1939 [5]. Cornual polyps are ectopic islands of normal endometrial tissue that arise within the interstitial part of the fallopian tube [6]. They are composed of both endometrial glands and stroma [3, 7, 8] and may manifest secretory change (Figure 2) [3, 5]. However, there is no invasion of the surrounding smooth muscle as occurs in adenomyosis or endometriosis. At HSG, polyps are seen as oval- or round- shaped filling defects, of 3–12 mm in diameter [9]. Conventionally, they are classified as small (<5 mm) and large (≥5 mm) [2]. They usually do not obstruct the fallopian tubes but rather are seen as filling defects within patent tubes at hysterosalpingography [10]. They occur unilaterally or bilaterally [2].

The reported frequency of associated infertility in patients with cornual polyps varies in the literature from 20% to 61.5% [11]. There is inherent bias since women who are infertile are more likely to have an HSG. There have also been sporadic reports of the association of cornual polyps with anovulation and/or endometriosis [1–3, 12]. We therefore wished to determine whether tubal polyps identified on HSG in infertile women in our tubal database are associated with either or both of these conditions. This is especially important given the paucity of data in the literature with respect to these associations and the implications for fertility treatment.

2. Materials and Methods

This case series was extracted from the tubal surgery database at the British Columbia Women's Hospital and The University of British Columbia in the period from January 1981 to December 2010. All standardized demographic and clinical data considered to be relevant to tubal infertility

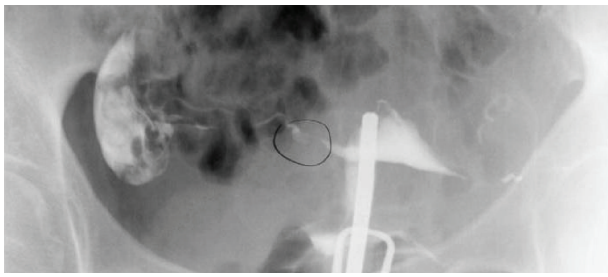

Figure 1: Hysterosalpingography. Circle indicates large right-sided tubal cornual polyp. Note tubal patency.

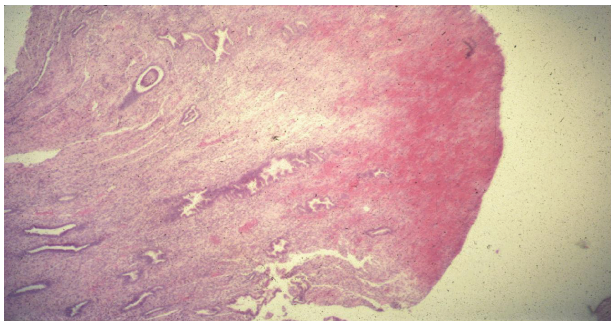

Figure 2: Light microscopy. Hematoxylin and eosin stain of cross-section of a large cornual polyp. Note secretory changes within endometrial glands. Infarction is evident at the right extremity of the polyp. Magnification ×55.

are entered prospectively into this ongoing administrative database system (dBase III Ashton-Tate, Culver City, Calif, USA) [13]. Infertility was defined by a period of at least 12 months of unsuccessful attempts at conception. The HSGs were performed predominantly at a single radiology centre and the images reviewed by one of the authors (P.F.McComb). Tubal cornual polyps were diagnosed in infertile women who had undergone an HSG. They were subclassified as large (≥5 mm) and small polyps (<5 mm). All polyps were removed from the cornua by microsurgery at laparotomy. The consecutive pattern of case accrual over time was bimodal. This reflected a belief by one of us (P.F.McComb) that this tubal surgery was of benefit in the early 1980s, and once again in the late 2000s, with an intervening period of equivocation.

In addition to the HSG, all women underwent a laparoscopy and assessment of ovulatory status. The diagnosis of endometriosis was based upon tissue biopsy or overt visual evidence at the laparoscopy. Anovulation was defined by luteal phase progesterone levels <5 nmol/L and/or by proliferative changes devoid of any secretory activity on endometrial biopsy.

The database also allowed identification of other potential infertility factors including male factor infertility.

Using the Statistical Package for the Social Sciences for Windows, version 19.0, categorical variables were evaluated by Fisher's exact test. A value of $P < 0.05$ was considered statistically significant.

3. Results

Twenty-two women with cornual polyps were referred for infertility evaluation and therapy. Seven women (mean age: 31.7 years; range: 26–40 years) had large cornual polyps (Table 1). Their mean duration of infertility was 46 months (range: 12–84 months). Only one person had neither anovulation nor endometriosis. Four women were anovulatory or oligoovulatory; in each case the cause was polycystic ovarian syndrome. Endometriosis was present in four women. Two women had both anovulation and endometriosis. Five of these women with large polyps also had either intrauterine endometrial polyps and/or prominent endometrial folds.

Fifteen women (mean age: 31 years; range: 25–38 years) had small cornual polyps. The mean duration of infertility was 47 months (range: 15–180 months). None of the 15 women with small tubal cornual polyps had coexisting anovulation; two had coexisting endometriosis. One had stage 1 and the other stage 3 endometriosis according to the revised American Society for Reproductive Medicine (ASRM) [14].

The concurrence of anovulation with large polyps versus small polyps was statistically significant ($P = 0.004$). The association between large polyps versus small polyps and endometriosis approached statistical significance ($P = 0.053$).

4. Discussion

This is the first series from a prospective tubal database to study the associations between tubal cornual polyps and anovulation and endometriosis. The limitations of this study are that it was not controlled, blinded, nor randomized. Nevertheless, the strong associations of these conditions with large cornual polyps are clinically meaningful.

Tubal cornual polyps may cause infertility in a variety of ways.

The pseudostratified endosalpinx of the intramural tube is characterized by a relative abundance of secretory cells [15]. This segment has three muscle layers: an outer spiral-longitudinal layer, which blends with the myometrium, and circular and inner longitudinal layers which penetrate as a distinct core deep into the myometrium [16]. The luminal diameter is 0.5 mm or less. The inherent intramural myosalpingeal contractility is similar to that of the neighboring isthmus, but acts upon a narrower and more deviating lumen than that of the isthmus [16]. By modulation of this contractility, the intramural portion of the fallopian tube initially retains the embryo within the isthmus of the fallopian tube for up to 3 days after ovulation and then releases the embryo into the endometrial cavity.

A large cornual polyp can perturb the structure and physiology of the intramural oviduct by dilatation of the lumen severalfold, alteration of muscular contractility, and/or the presence of the ectopic endometrium within the tube (with mucosa and glandular secretion that differs from that of the pseudostratified endosalpinx). We have also observed infarction of the distal extremity of these polyps at surgery (Figure 2); this may also interfere with embryo nurture and

Table 1: Clinical characteristics of infertile women with large cornual polyps.

Case	Age in years	Duration of infertility (months)	Polyp(s) size (mm)	Uterine cavity (HSG)	Anovulation	Endometriosis	ASRM stage endometriosis
1	34	59	Bilateral 3 × 8	Normal	Yes	No	N/A[a]
2	27	12	Bilateral 8 × 4	Normal	No	Yes	1
3	37	60	Right: 4 × 3 Left: 5 × 2	Polyp	No	Yes	1
4	40	84	Right: 4 × 2 Left: 6 × 4	Endometrial polyp	Yes	Yes	1
5	26	20	Right: 9 × 2 Left 12 × 3	Prominent endometrial folds	Yes	Yes	1
6	28	59	Bilateral 13 × 4	Endometrial polyps, endometrial fold	No	No	N/A[a]
7	30	28	Right: 6 × 2 Left: 5 × 2	Prominent endometrial fold	Yes	No	N/A[a]

[a]N/A: not applicable.

Table 2: Summary of published reports of the association of cornual polyps with anovulation (ovulatory dysfunction) and endometriosis.

Study	Year	Number of cases reported	Number of cases with anovulation (ovulatory dysfunction) and/or endometrial hyperplasia	Number of cases with endometriosis
Zenisek [12]	1959	10	10	0
Fernstroem and Lagerloef [3]	1964	17	5	4
David et al. [2]	1981	54	3	0
Gaudefroy et al. [17]	1970	47	10	0
McLaughlin [18]	1984	1	1	1
Glazener et al. [19]	1987	31	0	0
Stangel et al. [1]	1981	1	1	0
Philipp and Huber [5]	1939	4	0	4
Gordts et al. [7]	1983	52	Unstated	18
Gillett [8]	1989	5	0	5

transport. Infarction of large cornual polyps has also been reported by Bret and Grépinet [11]. It should be noted that cornual polyps do not appear to prevent the passage of contrast medium through the fallopian tube [10].

The reported frequency of an association of tubal polyps with infertility varies from 27% to 62% [2]. However, it remains unclear as to whether there is a direct causal relationship. This becomes especially tenuous when endometriosis and anovulation are potentially at play.

The association between tubal cornual polyps and anovulation and endometriosis has previously been reported in the literature [11] (Table 2). The polyp dimension is not always stated so that it is difficult to arrive at any consensus. In our series it is the large (bilateral) polyps that associate strongly with anovulation and endometriosis.

Zenisek [12] concluded that tubal polyps were caused by endometrial hyperplasia and found hyperplastic eutopic endometrium in all 10 of his tubal polyp cases. Five of the 17 patients with polyps reported by Fernstroem and Lagerloef [3] had glandular cystic hyperplasia (2 women) or polypoid hyperplasia (3 women) on endometrial biopsy. David et al. [2] found that 39% of women who had tubal polyps were anovulatory. Anovulation was the most common attributable cause of infertility in their group. A higher prevalence of anovulation in conjunction with cornual polyps has also been reported in two French studies [11, 17]. Furthermore, McLaughlin et al. [18] and Stangel et al. [1] each reported a woman with cornual polyps who was anovulatory. Logically, some have proposed that treatment of the coexisting anovulation would significantly increase the prospects for conception [1].

An association between cornual polyps and endometriosis has also been previously suggested. A summary of reported cases is shown in Table 2. Historically, in 1939, Philipp and Huber postulated that cornual polyps may cause endometriosis by discharging and spreading endometrial tissue via the fallopian tubes [5]. Fernstroem and Lagerloef reported four cases of ovarian and peritoneal endometriosis observed at laparotomy of their 26 cases of tubal polyps [3]. They concluded that there is presumptive evidence that women with tubal polyps have an increased tendency to develop ovarian or peritoneal endometriosis. Lisa and coworkers studied the intramural portions of the fallopian tubes in 300 posthysterectomy uteri [4]. They were the first

to show that cornual polyps are composed of endometrial tissue. Furthermore, they documented associated endometriosis at other pelvic sites. These authors concluded that the presence of endometrial tissue within the tubes appeared to be a normal developmental phenomenon. All 5 patients with tubal polyps reported by Gillett [8] had pelvic endometriosis. In the series reported by Gordts et al. [7], 41% of the 44 patients with cornual polyps who underwent laparoscopy had endometriosis. The anovulatory infertile woman with bilateral cornual polyps that McLaughlin [18] reported also had stage 2 endometriosis. Glazener et al. found no association with either endometriosis or anovulation, but it is unclear as to whether all their women with cornual polyps underwent laparoscopy to diagnose endometriosis and/or had assessment of ovulation [19].

We hypothesize that the coexistence of anovulation and endometriosis may lead to cornual polyp formation because both conditions are associated with proliferation of endometrium and/or formation of endometrial polyps. In our series, 5 of the 7 women with large cornual polyps had either endometrial polyps and/or prominent endometrial folds.

Some anovulatory syndromes, such as polycystic ovarian syndrome, predispose to estrogen-stimulated endometrial proliferation and hyperplasia [20, 21].

Women with endometriosis often form endometrial polyps [22]. Within the eutopic endometrium of women with endometriosis, there are multiple biochemical derangements. These include an increase in cycloxygenase-2 (COX-2) activity and aromatase activity, with overproduction of estrogen, prostaglandins, and cytokines [23]. Cellular proliferation is increased, and levels of apoptosis-related proteins are decreased [24]. Recently, it has been shown that endometriosis can be reliably diagnosed by the detection of increased nerve fiber density by immunohistochemistry in eutopic endometrium throughout the menstrual cycle [25, 26]. Such functional alterations, or abnormalities, in eutopic endometrium may predispose to endometrial polyp formation, and to cornual polyp formation.

In conclusion, the results of our case series confirm previous reports that infertile women with cornual polyps of the fallopian tube are more likely to have associated endometriosis and/or anovulation. It is possible that proliferative endometrial development and formation of endometrial polyps that may attend anovulation and endometriosis also predispose to the formation of cornual polyps. However, future studies with larger sample sizes are needed to better delineate this association.

Our findings of multiple infertility factors associated with tubal cornual polyps suggest two alternative therapies. Either IVF therapy and/or microsurgery to remove both the cornual polyps and ablate endometriosis, followed by induction of ovulation in anovulatory women.

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