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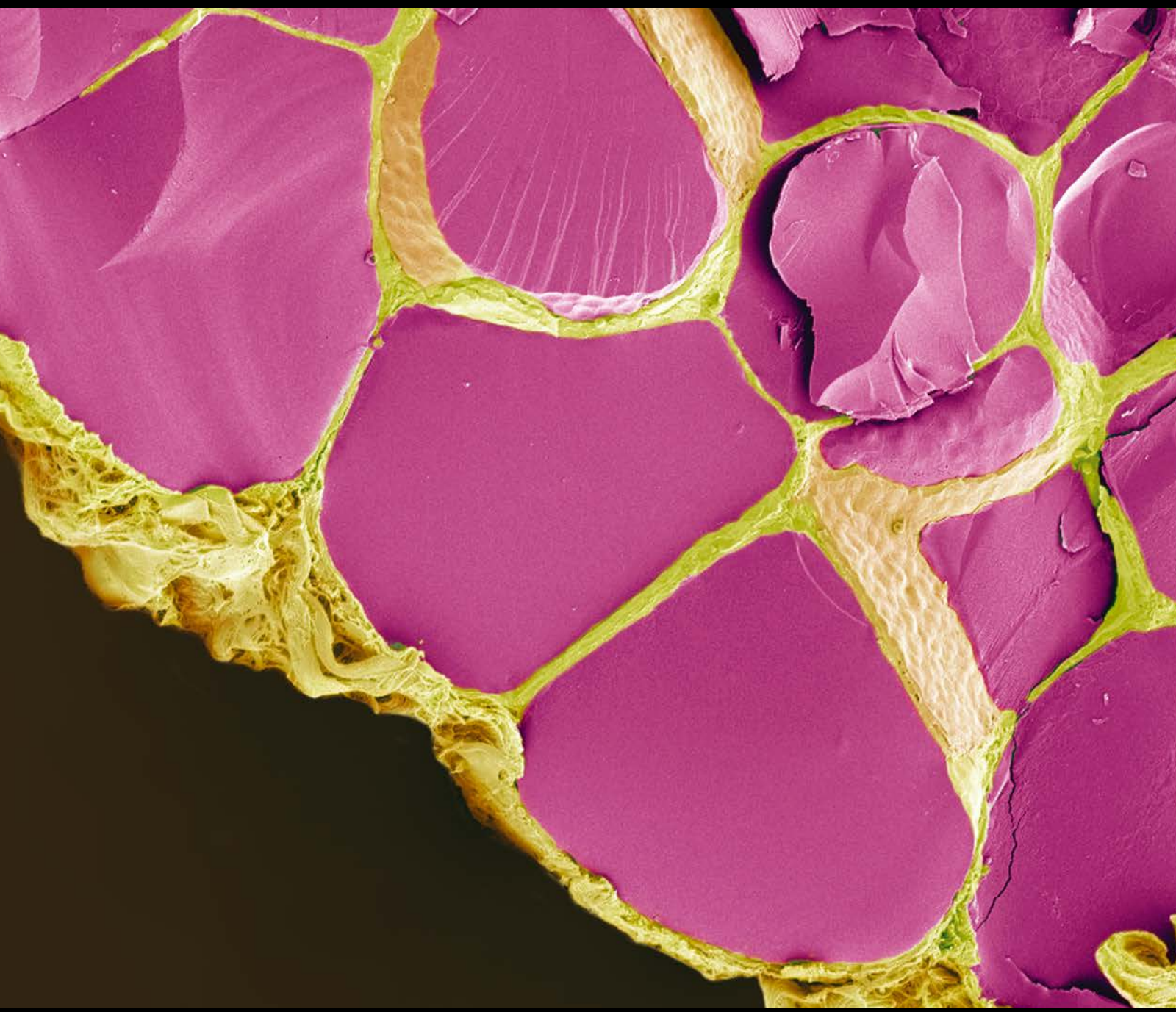
Inositol(s) from Bench to Bedside in Endocrinology and Gynecology

Guest Editors: Vittorio Unfer, John E. Nestler, Zdravko A. Kamenov, Nikos Prapas, and Fabio Facchinetti



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Editorial Board

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Editorial

Inositol(s) from Bench to Bedside in Endocrinology and Gynecology

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This special issue is aimed at providing innovative scientific data and discussing in-depth results and findings so far available to further clarify some pivotal scientific topics in the field of inositol(s). The authors focused their attention on three essential molecules belonging to this primordial group of ubiquitary chemical compounds: myoinositol and D-chiroinositol, two stereoisomeric forms of inositol(s), and inositol hexakisphosphate, a metabolite of myoinositol. These molecules are involved in many physiological functions and can be used for numerous therapeutic aims, in some cases exploiting their feature to exist in different enantiomeric forms, each one with specific tasks. Plenty of experimental and clinical data have shown that myoinositol alone, or (depending on the therapeutic aim) in association with D-chiroinositol in a physiologic ratio (40 : 1) between the two molecules, plays a pivotal role in treating several pathologies such as PCOS, metabolic syndrome, gestational diabetes, thyroid disorders, and infertility. Furthermore, myoinositol allows also improving substantially the outcomes of assisted reproduction technology (ART). Finally, myoinositol and inositol hexakisphosphate have demonstrated very promising anticancer activities as shown by numerous studies.

Although an increasing number of researches and investigations were realized on inositol(s) physiological properties as well as on the pathological conditions caused by their imbalance, a few points had to be reaffirmed and examined in-depth. The effort by the authors to address these issues was stimulated by the necessity to clear up a couple of improper ideas about the physiological functions carried out by myoinositol and D-chiroinositol. As a matter of fact, we may say that so far, not enough attention has been paid to this issue, at least in some cases. Despite the fact that it was well shown that such molecules play basic but very different roles in regulating various metabolic pathways and hormonal signaling, a few proposals of therapeutic application reveal to be still not appropriately focused. It is essential to highlight that myoinositol, the most represented and important stereoisomeric form of inositol, stands out for its key roles as leading molecule in some physiological areas, whereas different integrative functions are carried out by D-chiroinositol, synthetized from myoinositol under the enzymatic control of an epimerase. To get straight to the point, whereas the activation of glucose transporters and glucose utilization take place under the regulation of myoinositol, glycogen synthesis is mainly controlled by D-chiroinositol. On the other hand, in the ovary, myoinositol regulates glucose uptake and FSH signaling, while D-chiroinositol modulates insulin-induced androgen synthesis [1, 2]. In physiological conditions, the intracellular pool of inositol(s) in human ovaries and testis contains 99% of myoinositol whereas the remaining part is D-chiroinositol [2, 3]. It means that myoinositol is essential

qualitatively and quantitatively for the functioning of the reproductive system.

It was discovered that an imbalance between myoinositol and D-chiroinositol concentrations occurs in the ovary of PCOS women, with a myoinositol deficiency, which might impair the FSH signaling [2, 3]. In these conditions, glucose uptake and metabolism in oocytes and follicular cells are negatively affected, thereby compromising oocyte quality that depends on the availability of adequate amounts of myoinositol [2, 3]. The improvement of ovarian function, as well as hormonal and metabolic parameters, was demonstrated after myoinositol treatment in PCOS women [2, 3]. On the other hand, high doses of D-chiroinositol alone, administered to PCOS subjects, were found significantly detrimental for oocytes and therefore for fertility [2, 3]. Finally, it is not to be forgotten that myoinositol is a well-established safe molecule [4]. By now, it is possible to outline a comprehensive framework and bring about the best treatment for PCOS in keeping with the above cited findings, specifying some milestones useful for the therapeutic application. Many articles of the special issue contributed to further shed light on this topic and strengthen several points with original studies and insightful reviews of up-to-date scientific literature. In our systematic review (V. Unfer et al.), we analysed recent randomized clinical trials of inositol(s) in PCOS, in particular myo- and D-chiroinositol, aiming at better elucidating their physiological involvement in PCOS and potential therapeutic use, alone and in conjunction with assisted reproductive technologies, in the clinical treatment of women with PCOS. Other articles reported the results of a study on the putative role due to D-chiroinositol and its messenger in insulin resistance in women with PCOS independent of obesity (K. I. Cheang et al.) and an in-depth investigation on the possible involvement of inositol messengers in pre-eclampsia (S. Kunjara et al.). Other contributions were focused on PCOS therapy (and in a lesser extent on gestational diabetes and metabolic syndrome therapy), regarding the improvement of the metabolic profile and also the restoration of patients' fertility (A. S. Laganà et al., G. Muscogiuri et al.). On the same topic besides the cited reviews, various clinical studies are presented and carried out in adults (E. Benelli et al., A. C. Ozay et al., and S. Salehpour et al.) and teenagers (L. Pkhaladze et al.). Myoinositol was proven to be efficacious in increasing significantly the overall rate of live births in female mice (N. Kuşcu et al.), and in treating infertility in men, with or without metabolic syndrome (two studies by M. Montanino et al., M. Palmieri et al.), and in women affected or not by PCOS (D. Garg et al., B. Lesoine et al., P.-A. Regidor and A. E. Schindler, G. Simi et al., S. G. Vitale et al., and A. Wdoviak). Myoinositol, in association with folic acid, administered during pregnancy, was found to be very efficacious in warding off the occurrence of neural tube defects in infants (P. Cavalli and E. Ronda). Furthermore, other studies and reviews investigated the promising effects of myoinositol and selenium in the treatment of autoimmune thyroiditis (M. Nordio and S. Basciani), myoinositol plus D-chiroinositol, at the physiological ratio (40 : 1), for the therapy of type 2 diabetes (B. Pintaudi et al.) and for improving insulin resistance in obese male children (M. Mancini et al.). Also, myoinositol and its metabolite, inositol hexakisphosphate, deserve to be carefully taken into consideration. Both these molecules exert a wide range of critical activities in physiological and pathological settings. Deregulated inositol(s) metabolism was observed in a number of diseases, including cancer, where they modulate different critical pathways. Inositol hexakisphosphate was found to inhibit growth and invasiveness of several cancer types, whereas both inositol hexakisphosphate and myoinositol exert substantial chemopreventive effects in vitro and in vivo. Of note, myoinositol is able to significantly synergize with inositol hexakisphosphate in inducing cancer inhibition. Moreover, they are provided with antioxidant activity, moderate in myoinositol, and very high in inositol hexakisphosphate. The second one acts as a chelator of harmful trace elements, such as iron, uranium, nickel, copper, and other potentially toxic elements, often involved in tumor onset. In addition to that, these inositols modulate processes such as mRNA transcription, chromatin remodeling, cytoskeleton configuration, and p53 activity, to name a few. Thereby, inositol hexakisphosphate or myoinositol alone, or in synergy, can be excellent therapeutic agents that protect from cancer and other threats to human health (M. Bizzarri et al., R. Lauretta et al.).

We outlined the framework in which our special issue is placed, providing a guiding thread for reading the various contributions according to the topics covered by the authors. These articles convey a clear picture and can elicit new insights in researchers and readers concerning the studies on inositol(s).

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Fabio Facchinetti

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Review Article

Inositol and In Vitro Fertilization with Embryo Transfer

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Recently, studies on inositol supplementation during in vitro fertilization program (IVF) have gained particular importance due to the effect of this molecule on reducing insulin resistance improving ovarian function, oocyte quality, and embryo and pregnancy rates and reducing gonadotropin amount during stimulation. Inositol and its isoforms, especially myoinositol (MYO), are often used as prestimulation therapy in infertile patients undergoing IVF cycle. Inositol supplementation started three months before ovarian stimulation, resulting in significant improvements in hormonal responses, reducing the amount of FSH necessary for optimal follicle development and serum levels of 17beta-estradiol measured the day of hCG injection. As shown by growing number of trials, MYO supplementation improves oocyte quality by reducing the number of degenerated and immature oocytes, in this way increasing the quality of embryos produced. Inositol can also improve the quality of sperm parameters in those patients affected by oligoasthenoteratozoospermia.

1. Introduction

Despite 30 years of history, assisted reproduction technologies (ART) still present many challenges in order to identify factors and predictors of success.

It is well known that oocyte quality is the main factor determining chance of pregnancy and that poor quality is an obstacle for successful in vitro fertilization results. It has become increasingly clear that the follicular microenvironment of a human oocyte is a crucial factor for its developmental competence [1].

Through the years, many studies have been proposed to find strategies, drugs, or compounds such as antioxidant drugs and supplementation with vitamins or hormones able to improve oocyte quality and embryo quality [2].

Recently, studies on inositol supplementation during in vitro fertilization program (IVF) have gained particular importance due to the effect of this molecule on reducing insulin resistance improving ovarian function, oocyte quality, and embryo and pregnancy rates and reducing gonadotropin amount during stimulation [3]. Inositol and its isoform, especially myoinositol, find their application as prestimulation therapy in polycystic ovary syndrome (PCOS) patients undergoing IVF cycle and, recently, also in all kinds of infertile patients such as poor responders [4].

Studies demonstrate that the use of inositol in male patients affected by oligoasthenoteratozoospermia can improve sperm cell parameters and consequently the impact of fertilization rate and embryo quality leading to high percentage of pregnancy [5].

2. Inositol

Inositol (cyclohexanehexol) is a cycle polyol commonly referred to as a B vitamin, although not a true vitamin. It is widely distributed in human tissue and cells, and it is a precursor for phosphorylated compounds known as phosphoinositides which are involved in signal transduction through membrane receptor stimulation and other secondary messengers including diacylglycerol (DAG) and inositol triphosphate (IP3) that can be located at the inner or outer side of membrane and are involved in insulin transduction signaling. DAG activates protein kinase C (PKC) and IP3 activates intracellular calcium (Ca2+) release, an essential step in oocyte maturation and so of fertilization process. There are nine inositol stereoisomers, and myoinositol is the most represented in cellular content [6]. All stereoisomers act as mediator of insulin action inside the cell [7]: myoinositol (MYO) and D-chiro-inositol (DCI) are inositol-containing

phosphoglycan (IPG) mediators, generated by hydrolysis of glycosylphosphatidylinositol that inhibits cyclic AMP-dependent protein kinase (the first) and activates pyruvate dehydrogenase (the second one) [8].

MYO has been shown to influence different pathways at both ovarian and nonovarian levels. MYO is an important constituent of follicular microenvironment and it plays a determinant role in both nuclear and cytoplasmatic oocyte development [9], being also a precursor of phospholipids, which are responsible for the generation of important intracellular signals oocytes such as release of cortical granules, inhibition of polyspermy, and resumption of meiotic process [10]. Furthermore, MYO seems to significantly modulate steroidogenesis by acting through an insulin-independent pathway that involves cytoskeleton rearrangements [11].

On the contrary, DCI alone is not able to make significant improvements in the ovarian cell functions, as its beneficial effects are mainly confined to the nonovarian tissue in which it may significantly inhibit the negative cellular consequences of hyperinsulinemia. However, both inositol isomers can be effectively used in the management of PCOS patients in a ratio corresponding to their physiological plasma ratio (40 : 1). This seems to exert a synergistic effect according to a multitargeted design [12].

According to DCI ovary paradox theory, an increase of epimerase function in the ovaries causes an increase of DCI level associated with a local MYO deficiency and poor oocyte quality [13] with a negative effect in FSH stimulation and in ovulation [14]. Finally, some studies observed that high dosage of DCI administration may damage oocytes [15].

3. Inositol and In Vitro Fertilization

During the last decades, researches have focused on the role of the two major inositol stereoisomers, MYO and DCI, in particular on the effects of the first on oocyte quality. Among the causes of infertility, PCOS patients undergoing ovarian stimulation are subjected to an increased risk of in vitro fertilization failure due to poor oocyte/embryo quality and/or risk of ovarian hyperstimulation syndrome (OHSS). One of the goals of ovarian stimulation in PCOS is to recover an adequate number of mature oocytes avoiding OHSS. Clinical trials show that MYO supplementation started three months before the onset of ovarian stimulation results in significant improvements in hormonal responses, reducing the international unit (IU) of FSH needed to an optimal follicular development and estradiol levels at the day of ovulation trigger; this leads to a reduced risk of ovarian hyperstimulation syndrome (OHSS) and a lower number of canceled cycles. As shown by growing number of trials, MYO supplementation positively correlates with the number of oocytes retrieved and more importantly with the good quality of these. This means a reduction in the number of degenerated and immature oocytes, with consequently increased quality of embryos produced after fertilization [3, 16–18].

This result, obtained in both PCOS patients and non-PCOS women, is confirmed in the observation of Chiu et al. [19], who found that the amount of gonadotropin used for ovarian stimulation during IVF cycles is reduced in women whose follicular fluid contains higher levels of MYO. For these reasons, myoinositol supplementation appears really promising in ART and its effect has also been tested in non-PCOS patients undergoing fertility treatment in ART.

4. Inositol and PCOS in IVF

Polycystic ovary syndrome is one of the most common causes of infertility affecting 5–10% of females in reproductive age [20]. Typically, PCOS is characterized by hyperandrogenism (extremely variable in its occurrence), ovarian dysfunction with chronic anovulation and irregular menstrual cycle, polycystic ovaries at ultrasound evaluation, and dermatological problems such as acne, hirsutism, and seborrhoea [21].

Nowadays, a lot of trials show the importance of impaired insulin sensitivity as reason for many PCOS symptoms [22]. It has been hypothesized that an abnormal insulin signal transduction may cause insulin resistance, which induces anomalous ovarian steroidogenesis [20, 21]. Current therapies try to improve insulin resistance in order to reduce hyperinsulinemia resulting in metabolic and ovary function improvement. Insulin sensitizers, such as metformin, represent first line of therapy, but recently also inositol and its stereoisomers, myoinositol and D-chiro-inositol, have gained a lot of importance. MYO and DCI improve metabolic and endocrine function in PCOS patients; in particular, they determine a reduction in systolic arterial pressure and a reduction of LH/FSH ratio, and in addition they reduce circulating androgen and prolactin levels, increasing insulin action and sex hormone-binding protein levels [23]. Studies underlined the importance of myoinositol in oocyte differentiation and inositol ability to improve fecundation with in vitro fertilization techniques in women with PCOS compared to supplementation with D-chiro-inositol, which can damage oocyte if administered at high dosage [13, 15].

Recent trials note that women with PCOS respond to DCI with an increase in ovarian activity and menstrual frequency [24, 25]. However, subsequent studies demonstrated that MYO is more active than DCI [3, 26, 27] leading to regular menstrual cycles [24, 27] and to improvement of oocyte quality [28].

MYO supplementation during IVF cycle in PCOS patients increases oocyte quality [3, 16, 17] and embryo quality [29] and consequently implantation rate [4]. A recent analysis of follicular fluid in PCOS patients shows a 500-fold reduction in the amount of MYO, associated with an increase of insulin resistance, hyperinsulinemia, and luteinizing hormone levels [30].

Our recent study evaluates the effects of MYO administration on hormonal parameters in PCOS. 50 overweight PCOS patients undergo hormonal evaluations and an oral glucose tolerance test (OGTT) before and after 12 weeks of supplementation with myoinositol. Patients are divided into two groups: one treated with MYO 2 g and folic acid 200 μg daily (Group A) and the other one receiving only folic acid 400 mg (Group B-controls). Ultrasound examinations and Ferriman-Gallwey score are also performed. We note that after 12 weeks of MYO administration plasma

LH, PRL, T, insulin FSH result/FSH resulting significantly reduced. Insulin sensitivity, expressed as glucose-to-insulin ratio and HOMA index that indicate the insulin resistance ([basal glucose] × [basal insulin]/22.5), results significantly improved after 12 weeks of treatment. Menstrual cyclicity is restored in all amenorrheic and oligomenorrheic patients, while no changes occurred in patients treated with folic acid. After twelve weeks of treatment, an IVF cycle is performed for each patient: in the study group, the duration of stimulation is lower than in control group (11.5_0.8 versus 12.6_1.1; p¼0.002) and also rFSH units used are fewer. 17b–E2 levels (1839_520 versus 2315_601; p50.002), evaluated at the ovulation trigger day, are lower in the MYO group. Moreover, pregnancy rate (bHCG positive) is statistically significantly higher in the treated group (60% versus 32%; p50.05) [9].

Recently, Pacchiarotti et al. tested the synergistic effect of myoinositol and melatonin in IVF protocols with PCOS patients in a randomized, controlled, double-blind trial. They randomly divided five hundred twenty-six PCOS women into three groups: controls (only folic acid: 400 mcg), Group A (a daily dose of myoinositol: 4000 mg, folic acid: 400 mcg, and melatonin: 3 mg), and Group B (a daily dose of myoinositol: 4000 mg and folic acid: 400 mcg). They evaluated oocyte and embryo quality, clinical pregnancy, and implantation rates. Patients take inositols from the first day of the cycle until 14 days after embryo transfer. They observed that myoinositol and melatonin enhance, synergistically, oocyte and embryo quality so these two can be integrated routinely in association with drugs for ovarian stimulation during IVF cycle [30].

It is well known that ovarian hyperstimulation syndrome (OHSS) is an important iatrogenic complication in ART and that PCOS patients are at an extreme risk for the development of OHSS [31]. Recent trials point out that MYO is effective in preventing OHSS in a way similar to metformin [32].

5. Inositol in Non-PCOS Patients in IVF

Few data are available, at the moment, about the effects of MYO supplementation on in vitro fertilization outcome in sterile patients not affected by polycystic ovary syndrome. Recently, Lisi et al. have examined the effects of inositol administration on oocyte and embryo quality in infertile women undergoing IVF cycle by conventional IVF or intracytoplasmic sperm injection (ICSI). One hundred non-PCOS patients aged under 40 years and with basal FSH < 10 mUI/ml undergoing ovarian stimulation are randomly divided into two groups: Group A treated with 400 μg of folic acid for the 3 months before and during rFSH administration and Group B treated with a daily dose of 4000 mg of myoinositol into two administrations/day in addition to 400 μg of folic acid for the 3 months before and during rFSH administration. Group B shows a reduction in the number of mature oocytes retrieved and in the amount of gonadotropins used, whereas implantation rate and clinical pregnancy rate are improved [4].

The effect of MYO supplementation on ovarian function has also been evaluated in poor responders patients [33] undergoing ICSI. The study involves 76 poor responders divided into two groups: 38 patients who have been assuming MYO (4 g) plus folic acid (400 μg) for the previous 3 months before the start (Group A) and 38 patients assuming only folic acid (FA) (400 μg) for the same period (Group B). Ovarian stimulation is carried out with a GnRH antagonist protocol in both groups. They do not observe any significant difference between the two groups regarding oestradiol level, but total rec-FSH units used are significantly lower ($p = 0.004$) and metaphase II (MII) oocytes rate is significantly higher ($p = 0.01$) in Group A. The ovarian sensitivity index is higher, reaching a statistical significance ($p < 0.05$), in the group of patients pretreated with MYO, showing an improvement in ovarian sensibility to gonadotropin. In conclusion, they suggest that MYO supplementation in poor responder patients results in an increase of the number of oocytes recovered in MII and of the gonadotropin ovarian sensitivity, suggesting a MYO role in improving ovarian response to gonadotropins. Hence, MYO seems to be helpful in poor responders undergoing IVF cycles [34].

6. Inositol in Sperm Cell

About the role of MYO in male reproduction, Chauvin and Griswold show that MYO concentration in the seminiferous tubules is higher than in serum [35]; moreover, MYO levels are increased by movement of spermatozoa through epididymis and deferent duct [36]. Patients affected by oligoasthenoteratospermia have spermatozoa totally covered by "amorphous fibrous material," which reduces sperm mobility. Colone et al. show that MYO administration could help to reduce the presence of this amorphous material [37]. MYO has also a crucial role in the osmoregulation of seminal fluid and as a consequence in sperm progressive motility and velocities [38]. Gulino et al. investigate the effect of MYO administration on semen parameters of male patients undergoing IVF cycles. They collect semen samples of 62 patients divided into three different groups: healthy fertile patients (Group A); patients with oligoasthenospermia (OA) (Group B); and control group (CTR). The first two groups receive administration of 4000 mg/die of MYO and 400 μg/die of folic acid for 2 months. Semen's volume and spermatozoa's number and motility are the parameters evaluated before and after treatment and before and after density-gradient separation. Spermatozoa concentrations are higher in both Groups A and B. In conclusion, they showed that MYO supplementation significantly improves semen's parameters both in patients with OA and in normal fertile men [5].

7. Conclusion

Nowadays, many studies demonstrate the positive effects of myoinositol in patients undergoing IVF cycle so it could be a predictive factor in improving ART outcomes. In particular, as revealed by a conference scientific committee, MYO improves both ovarian response to gonadotropins during IVF stimulation and oocyte and embryo quality.

Conflicts of Interest

The authors declare that this research is conducted in the absence of any commercial or financial relationship that can be a potential conflict of interest.

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Clinical Study

Treatment with Myo-Inositol and Selenium Ensures Euthyroidism in Patients with Autoimmune Thyroiditis

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Clinical evidences have highlighted the efficacy of myo-inositol and selenium in the treatment of autoimmune thyroiditis. Aim of this study was to further analyze the role of myo-inositol plus selenium (Myo-Ins-Se) in restoring a normal thyroid function of Hashimoto's patients with subclinical hypothyroidism. Eighty-six patients with Hashimoto's thyroiditis having thyroid-stimulating hormone (TSH) levels between 3 and 6 mIU/L, elevated serum antithyroid peroxidase (TPOAb) and/or antithyroglobulin (TgAb), and normal free thyroxine (fT_4) and free triiodothyronine (fT_3) levels were enrolled in the study: one hyperthyroid subject with TSH about 0.14 μU/ml was included in this trial as a single case. Patients were assigned to receive Myo-Ins-Se. TSH, TPOAb, and TgAb levels were significantly decreased in patients treated with combined Myo-Ins-Se after 6 months of treatment. In addition, a significant fT_3 and fT_4 increase, along with an amelioration of their quality of life, was observed. Remarkably, TSH values of the hyperthyroid patient increased from 0.14 μU/ml up to 1.02 μU/ml, showing a complete restoration of TSH values at a normal range. In conclusion, the administration of Myo-Ins-Se is significantly effective in decreasing TSH, TPOAb, and TgAb levels, as well as enhancing thyroid hormones and personal wellbeing, therefore restoring euthyroidism in patients diagnosed with autoimmune thyroiditis.

1. Introduction

During the last decades, a sharp increase in thyroid pathology took place in most countries. The reasons for that may be explained not only because we have a better ability to make precocious diagnosis but also because other factors may have contributed to that increase. In this view, genes play an important role, since an individual with a family history positive for thyroid problems has a significantly higher possibility of developing a pathology of the gland. Also, environment may contribute to the development of these pathologies such as radioactive accidents, pollution, and other iatrogenic illnesses, especially those correlated with autoimmunity. For example, in regions with severe selenium (Se) deficiency, a higher incidence of thyroiditis may be documented, due to a decreased activity of selenium-dependent glutathione peroxidase activity within thyroid cells. Selenium-dependent enzymes are also key elements in the regulation of the immune system. Therefore, even mild selenium deficiency may lead to the development and maintenance of autoimmune thyroid diseases. In addition, the so-called "constitutional factors," such as age and sex, may influence and facilitate the appearance of thyroidal pathologies [1–5]. Among the numerous illnesses, thyroiditis is the most frequent (roughly about 20% of all thyroidal diseases) and is divided as acute, subacute, and chronic. The autoantibodies against thyroid presence are a peculiar feature during the evolution of most of them. A downregulation of suppressor T-lymphocytes and the ensuing activity against thyroglobulin (TgAb) and thyroid-peroxidase (TPOAb), one essential for the production and storage of thyroid hormones and the other involved in hormone synthesis, respectively, appear to be the starting point of the autoimmune process. Once the inflammatory cascade has been activated and the mechanism

initiated, T-lymphocytes may trigger a production of specific antibodies by B-lymphocytes [6]. Oxidative stress has been shown to be responsible for the onset of these autoimmunity disorders. Hence, an increase of TPOAb and TgAb concentration is largely seen. Concentration of these antibodies, as well as thyroid morphology, and the ability of follicular cells to produce thyroid hormones may change during life. Anyway, their presence may cause continuous damage to the thyroid tissue, leading to a decrease in hormones production. In fact, in patients with thyroiditis, undergoing long-term follow-up, very often a decline towards hypothyroidism is seen [7].

Inositol is better known as a family of slightly different compounds derived by a C_6 sugar alcohol. Of the nine 1,2,3,4,5,6-cyclohexanehexol isomers, Myo-Ins is the far most representative, with other inositols (allo-, cis-, d-chiro-, l-chiro-, epi-, muco-, neo-, and scyllo-) being less known, except for d-chiro-inositol that has an important role in insulin signal transduction and insulin resistance [8]. Several studies demonstrated that Myo-Ins is the precursor of the synthesis of phosphoinositides, which are part of the phosphatidylinositol signal transduction pathway across the plasma membrane, via the second messenger 1,4,5-triphosphate that modulates intracellular Ca^{2+} release [9]. Therefore, it acts as a second messenger regulating the activities of several hormones, such as insulin, follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH) [10]. As far as TSH signaling is concerned, after the binding of TSH to its receptor on thyroid cell surface, it stimulates cell growth and differentiation, in addition to thyroid hormone synthesis. This binding with TSH receptors activates two postreceptor cascades: one involves adenylyl cyclase, leading to an increase of intracellular cyclic AMP and protein kinase A phosphorylation and also to an activation of cytosolic and nuclear target proteins; the other is inositol-dependent and involves the phospholipase C-dependent inositol phosphate Ca^{2+}/diacylglycerol pathway, resulting in a boost of hydrogen peroxide (H_2O_2) generation. In addition, while the cAMP pathway is more involved in cell growth, differentiation, and thyroid hormones (T_4-T_3) secretion, the inositol-dependent pathway regulates H_2O_2-mediated iodination of thyroglobulin.

TSH, a glycoprotein synthesized and secreted by the pituitary gland, regulates the release of thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4), from the thyroid. These hormones modulate many physiological processes in the human cells and are crucial for growth, development, differentiation, and the maintenance of basal metabolism [11]. Most scientists agree in defining the T_4 more like a "prohormone" rather than a real hormone, although it is the major hormone secreted by the thyroid [12]. T_4, in fact, is not very active; it expresses the functional activity of the gland, but to be useful to the human body it must be converted to T_3. Here, through the enzyme 5′ deiodinase, the prohormone is deprived of one iodine and, in this way, is activated. Only T_3 can enter easily into the tissue cells where it carries out its physiological functions. Hypothyroidism and hyperthyroidism are thyroid disorders that can be caused by TSH signal transduction impairment. Indeed, elevated thyroid-stimulating hormone (TSH) and autoantibodies levels, such as TgAb and TPOAb, are typical features of autoimmune thyroiditis. In such pathologies, among which Hashimoto's thyroiditis (HT) is the most representative, thyroid gland gets underactive, as attacked by cell- and antibody-mediated autoimmune processes [13]. Excluding HT, a mild thyroid failure is called subclinical hypothyroidism (SH) [14] and usually is balanced by a slight TSH level increase, varying in the range 3–6 mIU/L. According to the American Thyroid Association's (ATA's) guidelines, the normal range for TSH values, with an upper limit of 4.12 mIU/L, is largely based on National Health and Nutrition Examination Survey (NHANES) III data, but it has not been universally accepted. In fact, some have proposed that the upper normal TSH values should be either 2.5 or 3.0 mIU/L [15].

The relevant impact of Se on inflammatory activity in thyroid-specific autoimmune disease has already been shown in several trials [16–19], demonstrating its possible therapeutic effect in reducing TPOAb in patients with autoimmune thyroiditis (AIT). AIT like HT, idiopathic myxedema, and Graves' disease are characterized by the high levels of TPOAb (>50 lU/ml), which are closely associated with abnormal levels of TSH (<1.0 mIU/L in hyperthyroidism and >2.5 mIU/L in hypothyroidism forms) and correlated with progressive thyroidal damage and lymphocytic inflammation [20]. Graves' hyperthyroidism and Hashimoto's hypothyroidism might be the opposite spectrums of one disease. In a previous study of ours, the beneficial effect of Myo-Ins in reducing TSH levels through the improvement (increase) of TSH sensitivity was highlighted. Essentially, it was shown that supplementation of Myo-Ins-Se was able to restore the euthyroid state and improve personal wellbeing in subclinical hypothyroidism patients [21].

Taking into account our previous data, the aim of this study was to further investigate the efficacy of Myo-Ins-Se in restoring the euthyroid state of Hashimoto's patients with subclinical hypothyroidism. A single case of hyperthyroidism was also included in the study drawing attention to the unique therapeutic approach of Myo-Ins.

2. Patients and Methods

A total of 87 patients, 8 men and 79 women (mean age 42.30 ± 0.06 years), were included in our study; 86 were meeting the inclusion criteria as follows: age range 19–65, TSH levels between 3 and 6 mIU/L, elevated serum TPOAb and/or TgAb, and normal free thyroxine (fT_4) and free triiodothyronine (fT_3) levels. A hyperthyroid subject, a woman with TSH around 0.14 mIU/L at baseline, was also selected to enter the study. None of the patients were undergoing adjuvant treatment with trace elements, vitamins, or antidepressive and antipsychotic drugs. Patients were otherwise healthy. Informed consent was obtained from all participants in this study. Patients received tablets containing 600 mg myo-inositol plus 83 μg selenium in the form of L-selenomethionine (Tiroxil®, Lo.Li. Pharma Srl, Rome, Italy) orally for 6 months. Participants were asked to take the medication with water about 2 h before or after meal. Primary outcome was detection of serum TSH levels.

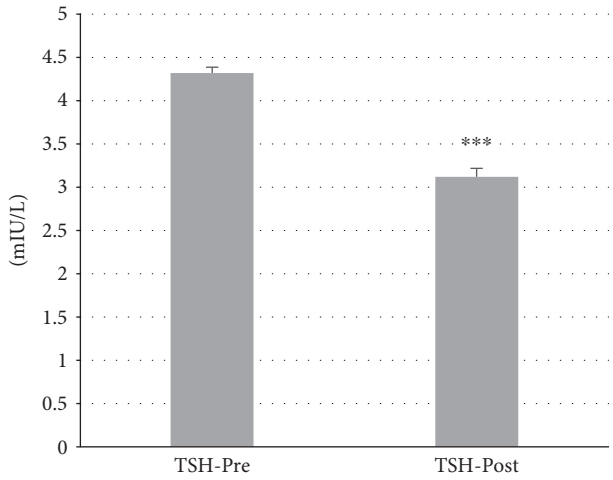

Figure 1: Thyroid-stimulating hormone (TSH) levels in patients at baseline and after 6 months of Myo-Ins-Se treatment ($n = 86$). Values are expressed as median (±SEM). Comparison of TSH levels at baseline versus posttreatment, $^{***}p \leq 0.001$.

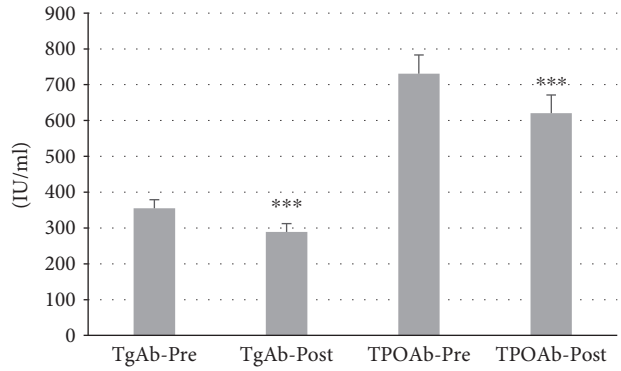

Figure 2: Serum antithyroglobulin (TgAb) and antithyroid peroxidase (TPOAb) levels of patients at baseline and after 6 months of Myo-Ins-Se treatment ($n = 86$). Values are expressed as median (±SEM). Comparison of TgAb and TPOAb levels at baseline versus posttreatment, respectively; $^{***}p \leq 0.001$.

Secondary outcomes were TPOAb, TgAb, fT_3, and fT_4 hormone concentrations as well as quality of life evaluation.

2.1. Laboratory and Technical Investigations. The investigation was performed over a period of 6 months. Blood samples were drawn from each patient, and serum TSH, fT_3, fT_4, TPOAb, and TgAb levels were measured at baseline and at the end of the study. TSH, fT_3, and fT_4 concentrations were measured by an enzyme immunometric assay (Byk-Sangtec Dietzenbach, Germany). Plasma total TPOAb and TgAb concentrations were measured by a commercial enzyme luminescence assay (Byk-Sangtec, Dietzenbach, Germany). At enrolment and after 6 months, the subjective symptomatology (SS) was evaluated using a questionnaire, including 7 questions testing the presence of symptoms; in particular, the SS comprises local symptoms such as pain localized on the front of the neck, discomfort when swallowing liquids or solids, ability to raise the voice, and feeling "constriction" in the neck wearing high-necked clothes and accessory-type choker necklaces or when lying down [22].

2.2. Statistics. Data were processed using paired *t*-tests, with GraphPad Software (GraphPad Software Inc., La Jolla, CA, United States). Values are expressed as median (±SEM), and a p value ≤0.05 was utilized throughout as a criterion for any result that was statistically significant.

3. Results

In total, 87 patients with autoimmune thyroiditis were enrolled in the study. The median age of patients was 42.30 ± 0.06 years (range: 19–65). All patients were receiving Myo-Ins-Se treatment for 6 months. A significant reduction of TSH levels was observed in patients' posttreatment, from 4.32 ± 0.06 mIU/L at baseline to 3.12 ± 0.09 mIU/L after treatment ($p \leq 0.001$) (Figure 1). There were significant decrements in both autoantibodies TgAb and TPOAb serum levels after administration of Myo-Ins-Se: TgAb levels decreased from 344.96 ± 23.77 IU/ml to 288.84 ± 23.35 IU/ml after treatment ($p \leq 0.001$) and TPOAb from 720.67 ± 52.39 IU/ml to 620.38 ± 50.90 IU/ml, pre- and post-Myo-Ins-Se treatment, respectively ($p \leq 0.001$) (Figure 2). The serum fT_3 and fT_4 levels of patients were slightly but significantly higher at the end of 6-month period when compared with the values at baseline: fT_3 values were 2.67 ± 0.04 pg/ml at baseline and 2.79 ± 0.03 pg/ml posttreatment ($p \leq 0.01$) and fT_4 levels were 0.94 ± 0.02 ng/ml and 1.07 ± 0.02 ng/ml ($p \leq 0.001$) pre- and posttreatment, respectively (Figure 3). In Figure 4 are shown the TSH values from each patient including also the hyperthyroid patient. The graph clearly shows how TSH levels decreased in Hashimoto's patients with SH but increased in the hyperthyroid patient from T0 to T1 (baseline and 6-month treatment, resp.) (Figure 4). In particular, TSH values of the hyperthyroid patient increased from 0.14 mIU/L up to 1.02 mIU/L, therefore restoring TSH values at a normal range (Figure 4). Questionnaire of SS revealed a significant improvement of patients' quality of life after 6 months of Myo-Ins-Se administration. Values dropped down from 4.67 ± 0.09 at baseline to 2.37 ± 0.09 at the end of treatment ($p \leq 0.001$) (Figure 5).

4. Discussion

The subject of the present study was to further examine the efficacy of Myo-Ins-Se in restoring the euthyroid state in patients affected by thyroid disorders. We could demonstrate that, in patients with AIT, the concentrations of TSH, TPOAb, and TgAb significantly decreased after administration of Myo-Ins-Se for 6 months. Furthermore, quality of life was significantly improved in all patients at the end of this study. A single case of hyperthyroidism was also analyzed, emphasizing the effect of Myo-Ins in increasing TSH levels up to normal concentrations.

Until recent years, the pharmacological approach to inflammatory thyroid pathologies, especially those having a high titer of autoantibodies, was based upon the use of corticosteroids that, of course, are able to temporarily decrease

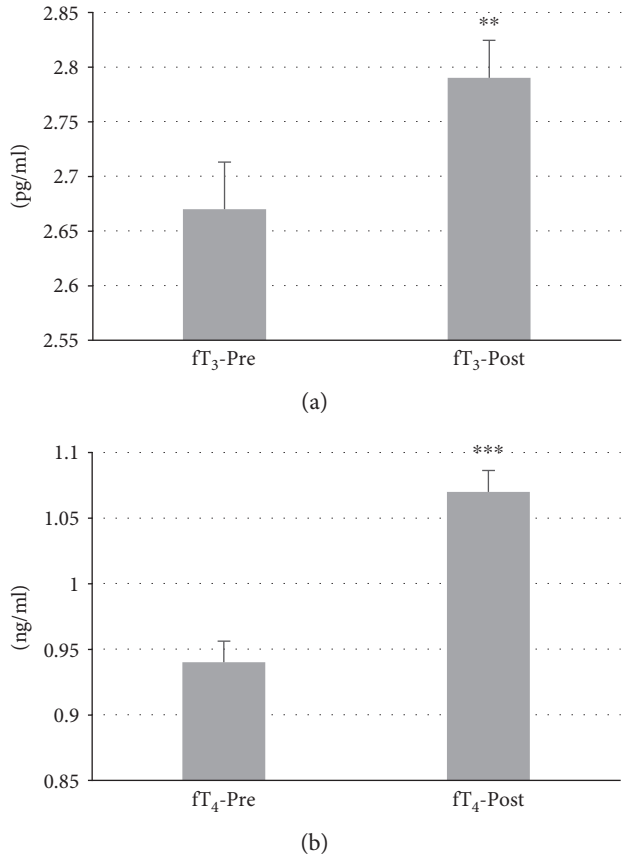

Figure 3: Free triiodothyronine (fT_3) and free thyroxine (fT_4) levels of patients at baseline and after 6 months of Myo-Ins-Se treatment ($n = 86$). Values are expressed as median (±SEM). Comparison of fT_3 (a) and fT_4 (b) concentration at baseline versus posttreatment, respectively; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

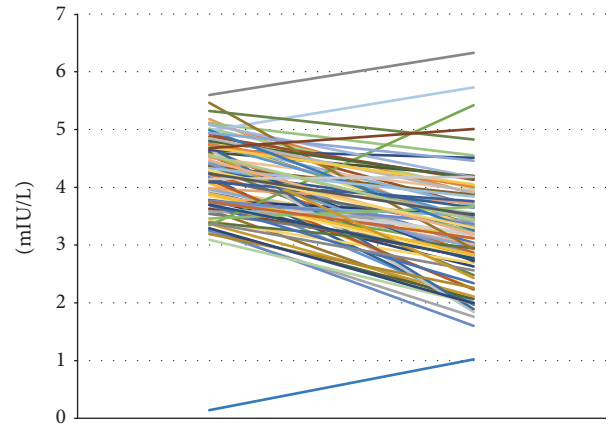

Figure 4: Thyroid-stimulating hormone (TSH) levels in patients at baseline (T0) and after 6 months (T6) of Myo-Ins-Se treatment ($n = 87$). Hyperthyroid patient is included in this graph.

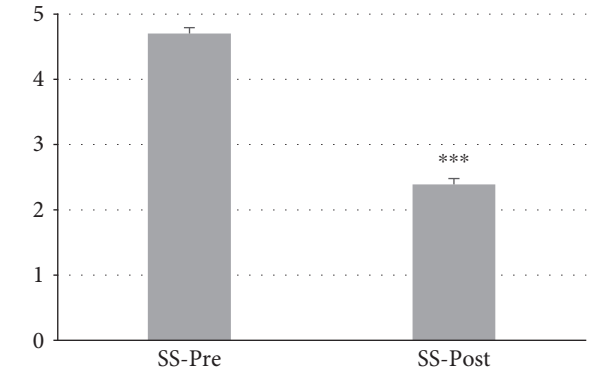

Figure 5: Subjective symptomatology of patients at baseline and after 6 months of Myo-Ins-Se treatment ($n = 87$). Values are expressed as median (±SEM). Comparison of patients' subjective symptomatology at baseline versus posttreatment; $^{***}p \leq 0.001$.

inflammation and antibody concentrations [23, 24]. However, being almost the only way of intervention (FANS are also used), they have been frequently overused, with the consequence of an increase in the percentage of their well-known adverse effects. In this view, a series of compounds able to ameliorate SS, inflammation parameters, and thyroid status have been identified to date. Among them, inositol and Se seem to be the most efficacious in terms of thyroid function recovery and symptomatology amelioration. The story of inositol is a long one, since it started around the last two decades of the previous millennium, when researchers demonstrated its ability as a calcium-mobilizing second messenger [25] and to decrease insulin resistance in polycystic ovary syndrome [26]. Therefore, a widespread research activity was initiated, giving rise to an important series of information, clarifying various aspects of hormonal signal transduction. In particular, it has been shown that relatively low TSH concentrations are able to stimulate cAMP-mediated signal cascade, while only a 100-fold higher TSH concentration is needed to stimulate the inositol-mediated signal cascade [27]. Therefore, it can be speculated that impairment of the inositol-dependent TSH signaling pathway may be, at least in part, one cause of thyroid malfunctioning and that, by increasing the availability of Myo-Ins at cellular level, it is possible to improve TSH sensitivity of the thyroid follicular cell. In fact, previous clinical studies, as well as this one, indicate that the administration of 600 mg Myo-Ins is able to ameliorate thyroid function and symptomatology in patients with HT [21].

The physiological role of TSH is quite crucial in the regulation of hypothalamic-pituitary-thyroid axis, as it modulates the release of the thyroid hormones from the thyroid gland. It prompts iodine uptake by the thyroid [28], induces thyroid epithelial differentiation and growth [29], and preserves thyroid cells from apoptosis [30]. In fact, impairment of TSH signal transduction can lead not only to thyroid disorders such as hypothyroidism and hyperthyroidism but also to proliferation and differentiation of human thyroid carcinoma cells [31]. TSH increase is also associated with a higher risk for coronary heart disease (CHD) accidents, increased CHD mortality, and heart failure (HF) events, particularly in those patients with TSH levels >10.0 mIU/L [32, 33].

In this study, it is shown how Myo-Ins acts on TSH levels lowering them when too high and increasing them if too low.

Even lowering the TSH, the production of thyroid hormones, T_4 and T_3, is induced. As mentioned above, normally, TSH induces the uptake of iodine by the thyroid gland as well as the production of thyroid hormone; increased levels of TSH stimulate the thyroid to produce more thyroid hormones, thereby returning the level of thyroid hormone in the blood back to normal. Our study shows that, after Myo-Ins-Se supplementation for 6 months, TSH levels significantly decreased in HT's patients with SH without affecting the production of thyroid hormones that, as a matter of fact, were significantly increased after the treatment. Hypothyroidism results from a deficient production of thyroid hormone by the thyroid gland. Since the thyroid hormones regulate metabolism in every cell of the body, a shortage of them can virtually affect all body functions. Deficiency of thyroid hormones can result from lack of stimulation by the pituitary gland, a defective hormone synthesis, or the impaired cellular conversion of T_4 to T_3. Another substance widely used in the treatment of AIT is Se. In fact, as reported earlier, it is a trace element, essential to wellbeing, that exerts multiple and complex effects on human health [34]. The physiological functions of Se are carried out by selenocysteine, the 21st amino acid, which is the defining feature of the 25 selenoprotein-encoding genes so far discovered within the human genome [35]. Se can exert an influence on immunological responses, cell growth, and viral defense. It is an essential particle in the active site of enzymes such as glutathione peroxidases, deiodinases, and thioredoxin reductases. In addition, it has a fundamental importance in the synthesis and function of thyroid hormones and protects cells against free radicals and oxidative damage. In fact, Se demonstrates antioxidant and anti-inflammatory properties that have a relevant impact on immune function [36, 37] and it has been shown to reduce the inflammatory status in patients with HT [16]. Intake of Se necessary to maintain suitable selenoenzyme activity is about 55 μg per day. Se deficiency contributes to decreased activity of glutathione peroxidases, which can lead to oxidative damage, or deiodinase, which is connected with impaired thyroid activity. Moreover, a low Se concentration causes autoimmune processes in the thyroid gland; thus Se deficiency is essential to the pathogenesis of autoimmune thyroiditis or Graves' disease. A number of studies have shown indeed the effectiveness of Se in lowering the autoantibodies, in particular the TPOAb concentration in AIT's patients [16, 38, 39]. These findings are in line with our previous [21] and recent results where a significant decrease in serum TPOAb and TgAb levels was observed at the end of Myo-Ins-Se treatment. To be noted, different from the sole supplementation of Se, the combination Myo-Ins-Se is able to decrease not only TPOAb but also TgAb levels.

Therefore, the conclusion of our study is that the supplementation of Myo-Ins-Se is able to restore the euthyroid state as well as improve the wellbeing of Hashimoto's patients with SH. It would be of interest to further investigate the effect of this combined therapy in a more considerable group of hyperthyroid patients since, in this single case, we have obtained very prominent results, showing that Myo-Ins-Se restores TSH levels up to normal values. Bearing in mind also the safety of these two molecules' usage, accentuated by the absence of side effects, the Myo-Ins-Se combination can be considered a very efficacious and safe therapy for AIT treatment.

Competing Interests

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

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Review Article

Myoinositol: The Bridge (PONTI) to Reach a Healthy Pregnancy

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The use of folic acid in the periconceptional period can prevent about 70% of neural tube defects (NTDs). In the remaining cases, no medical prevention is available, and those conditions should be defined as folate-resistant NTDs. Rodent models suggest that some folate-resistant NTDs can be prevented by inositol (myoinositol and chiroinositol) supplementation prior to pregnancy. Should folic acid be combined with myoinositol periconceptional supplementation to reduce the overall risk of NTDs even in humans? Hereafter, we discuss the results from the PONTI study that strongly support both the effectiveness and safety of myoinositol periconceptional supplementation in preventing human NTDs. We further report on the largest case series of pregnancies treated with myoinositol and folic acid. At our institution, a sequential study during 12 years involved mothers at risk of fetal NTDs, and 29 babies from 27 pregnancies were born after periconceptional combined myoinositol and folic acid supplementation. No case of NTDs was observed, despite the high recurrence risk in the mothers. Taken together, those data suggest that periconceptional folic acid plus myoinositol can reduce both the occurrence and recurrence risks of NTDs in a greater number of cases than folic acid alone.

1. Background

According to EUROCAT (European Surveillance of Congenital Anomalies) data, the EU prevalence of major congenital abnormalities is 23.9 per 1000 births. Among those defects, the frequency of nervous system defects has been calculated at 2.3 per 1000, and the prevalence of neural tube defects (NTDs) is 0.9 per 1000 (years 2003–2007) [1]. The neural tube is an embryonic structure that gives rise to the Central Nervous System (CNS) and is completely closed in the fourth week of gestation. An incomplete or no closure causes a severe anomaly of the CNS, and the subsequent exposure to the amniotic fluid environment leads to neurodegeneration and to severe damage of the exposed brain and/or spinal cord [2].

In most European countries, prenatal diagnosis of NTDs is easily available by ultrasound examination, and about 70% of spina bifida and 83% of anencephaly cases are prenatally identified, most of them leading to termination of pregnancy [3, 4].

So, it should be argued that NTDs seemingly became an "invisible" problem, as rarely seen in the newborn. However, the problem related to NTDs is far to be resolved and comes back in through the window, since the need of estimating the risk of recurrence for the next pregnancies is required for each affected pregnancy and reducing that risk should be the goal of every medical intervention.

Periconceptional folic acid supplementation has proved able to reduce the risk of NTDs up to 70% (occurrence risk: OR 0.28, CI 95% 0.15–0.53; recurrence risk: 0.68, CI 95% 0.17–0.60) [5].

Folic acid fortification also reduces the risk of NTDs (OR 0.54, CI 95% 0.46–0.63) [6]. Given those data, it comes without saying that about 30% of NTDs should be considered "resistant" to folic acid fortification and supplementation, and no intervention is available in those cases that are resistant to folic acid intake. The present paper deals with the problem of preventing that particular class of NTDs (folate-resistant NTDs) through the therapeutic use of inositol, a cyclic carbohydrate with a 6-carbon ring structure. Inositol is found widely throughout mammalian tissues as phosphatidylinositol and in cell membranes as phosphoinositide. It is no longer considered a member of the vitamin B complex, as it was in the past, since it can be produced by the human body from glucose. Inositol is a fundamental nutrient required by human cells in culture to grow and survive.

2. Inositol and Neural Tube Defects: Experimental Models

The relation between NTDs and inositol was first suggested in 1988 in rat embryos [7]. After that preliminary observation, the role of inositol in embryonic development and its effects on embryogenesis were further explored in the early 1990s, using rodent models. The supplementation with inositol of mouse embryo cultures exposed to an excess of glucose was shown to reduce the incidence of neural tube defects, normalizing the closure of the anterior neural tube. Indomethacin, an inhibitor of cyclooxygenase-1 and cyclooxygenase-2 which are involved in arachidonic acid metabolism and prostaglandin synthesis, can reverse the favorable effects of inositol on neural tube closure. Moreover, the addition of prostaglandin E2 to those embryo cultures leads to the normal development and closure of the neural tube, suggesting a protective role of both inositol and arachidonic acid pathway against CNS defects associated with diabetic embryopathy [8]. At the same time, an inverse correlation between glucose concentration and inositol content in rat embryo cultures was demonstrated, as well as a significant reduction of neural defects after inositol supplementation [9]. Following those seminal observations, the relationship between inositol and NTDs was confirmed in curly tail mice a few years later [10]. The curly tail mutant mouse has been used since 1976 as an experimental model to study NTDs [11], as the homozygous mutant ct (ct−/ct−) mouse is associated with NTD phenotypes (spina bifida, curly tail, and exencephaly), although characterized by variable expression and incomplete penetrance.

Of note, inositol deficiency is associated with an increased frequency of NTDs in the curly tail embryos in vitro [12].

Spina bifida in curly tail mice (ct−/ct−) results from a defective closure of the posterior neuropore, which forms the caudal region of the neural tube. The balance between the anterior and posterior growths of embryonic tissues reflects on the angle of curvature of the body axis.

In (ct−/ct−) mice, both the posterior endoderm and the notochord grow slowly, and the unbalanced growth between ventral and dorsal tissue causes an excessive curvature of the caudal region, which in turn induces a delay in the closure of the posterior neuropore, resulting in the curly tail phenotype, or in more severe closure defect, represented by spina bifida. A balanced embryonic tissue growth can normalize the curvature of the body axis, thus reducing the frequency of spina bifida. In the curly tail model, the neural tube closure can be normalized by the administration of inositol, which stimulates the growth of mesodermal tissues, thus restoring the normal growth of the embryo [13, 14].

Further evidence of the preventive effect of inositol on NTDs incidence is not restricted to curly tail mice, since protection against diabetes-induced NTDs has been observed in other rodent models.

Maternal diabetes is a condition associated with an increased risk of fetal malformations, and among them, NTDs are often observed.

Dietary inositol supplementation of 0.08 mg/kg/day is also effective in significantly decreasing the frequency of embryonic NTDs in diabetic rats [15, 16]. Of note, no evidence of an adverse effect of inositol either on pregnancy success or on fetal outcome was found in inositol-treated mice.

A more comprehensive approach revealed that the incidence of NTDs in the curly tail mutant mouse is not reduced by folic acid, suggesting that different subtypes of NTDs exist, some of them being resistant to folate intake.

As stated above, no more than 70% of human NTDs can be prevented by folic acid administration, so the curly tail mutant mouse was viewed as an experimental model to test the possibility of a combined treatment of folic acid plus inositol even in humans.

More recently, other findings strongly suggest that inositol metabolism is needed for normal brain and CNS development and that NTDs are associated with disruption of inositol signaling [17, 18].

Therefore, data from rodent models strongly support a distinct inositol-dependent metabolic pathway that, when stimulated, can prevent the cellular dysfunction leading to spinal NTDs.

3. Inositol and Neural Tube Defects: Humans

As inositol supplementation significantly reduces the incidence of spina bifida in the curly tail mouse, as well as in diabetic rats, the question is whether the risk of NTDs could be reduced also in humans and whether the risk of folate-resistant NTDs can be reduced by inositol treatment [19].

In humans, significantly lower inositol concentrations have been reported in the blood of mothers carrying NTD fetuses compared with normal pregnancies, and mothers with low blood levels of inositol show a 2.6-fold increased risk of an affected offspring [20].

Moreover, the association between maternal diabetes and the risk of fetal NTDs can be explained by the competitive effect of hyperglycemia on the transmembrane transportation of both arachidonic acid and inositol, suggesting that inositol supplementation could restore normal levels of cellular myoinositol and counteract the inhibition of neural tube closure caused by elevated glucose blood levels [8].

To answer the question whether inositol could be useful in reducing the recurrence risk of NTDs in human pregnancies, Cavalli and Copp proposed the first periconceptional treatment with myoinositol and folic acid to a woman with two previous NTD-affected pregnancies despite a correct folic acid intake. The recurrence of those congenital malformations was attributed to the resistance to folic acid, likely mimicking the curly tail experimental model.

That woman underwent supplementation with 500 mg myoinositol and 5 mg folic acid in the periconceptional period, and a healthy baby was born. Of note, no adverse effects on the mother and the fetus were associated with that treatment [21].

However, the effectiveness of inositol intake in reducing NTD risk has been questioned, as dietary inositol intake and NTD risk were not correlated statistically in a retrospective questionnaire analysis [22]. Still, the results from a questionnaire analysis cannot be considered as reliable as the measurement of inositol in blood or urine and should

be taken cautiously. Moreover, it could be argued that inositol supplementation, but perhaps not normal dietary intake, could be beneficial in preventing folate-resistant NTDs in humans, as in rodents.

In any case, after the first pioneering result, a prospective pilot study was started aiming to demonstrate that women with at least one previous NTD-affected pregnancy despite correct folate intake and exposed to myoinositol in their periconceptional period (a) will not experience any adverse effects after inositol supplementation (1000 mg/day) and (b) will give birth to babies not affected by NTDs, so demonstrating a positive effect in reducing the recurrence risk.

Even in the absence of statistical significance, and given the difficulty to design and perform a randomized clinical trial (RCT), the prospective study demonstrated that myoinositol taken in the periconceptional period was not associated with collateral or side effects.

The study was performed on 15 pregnancies at risk of putative folate-resistant NTDs treated with inositol and folic acid (1000 mg myoinositol and 5 mg folic acid) in their periconceptional period, starting at least 60 days before conception and continued until the 8th week of pregnancy. All the women underwent correct folate supplementation in their previous affected pregnancies. Despite their high recurrence risk, all the babies were born without NTDs, and no adverse event was reported in that series [23].

4. Recent Results

Based on the results reported before, the PONTI (Prevention of Neural Tube Defects by Inositol) study was designed as a pilot study, aiming to demonstrate the feasibility of a randomized clinical trial (RCT) to evaluate the effectiveness of inositol plus folic acid in preventing a greater number of NTDs as compared to folic acid alone, according to the previous experimental hypothesis. The results from the PONTI study have been recently published [24].

The PONTI study confirmed the difficulty of enrolling the greater number of cases needed to perform an RCT, as well as the burden of obtaining significant results.

However, those results confirm a trend towards the association between inositol and folic acid intake and a reduced risk of NTDs.

Moreover, the PONTI study confirms the feasibility of a full-scale clinical trial on the use of inositol and folic acid to prevent a greater number of NTDs than folic acid alone.

Among the 117 women with a previous NTD-affected pregnancy and planning a new pregnancy that were contacted, 18 were ineligible, 52 declined randomization, 30 were lost at follow-up, and 33 were randomized for each arm (folic acid plus inositol versus folic acid plus placebo).

A corrigendum was added after publication, making the study results somewhat difficult to discuss. However, the final results are favorable and are described as follows:

(i) 14 women in the inositol arm gave birth to normal babies.

(ii) One affected pregnancy was found in the control group (19 originally randomized).

24 pregnancies were reported among the 52 women who declined randomization, and 2 NTDs were born from women not taking inositol. Again, no NTD case was reported in women who underwent inositol supplementation.

Those results confirm the association between inositol and folic acid intake and a reduced risk of NTDs.

Of note, no adverse events were found in all pregnancies treated with inositol, nor in the control group, suggesting that periconceptional inositol supplementation should be considered safe for both the mothers and the fetuses. In our opinion, this is one of the most important results of the PONTI study, and the safety of inositol intake prior to pregnancy should be underlined.

Further interesting results came from the evaluation of inositol measurement by mass spectrometry assay, suggesting that measuring blood inositol levels is more accurate than measuring urinary inositol, still allowing monitoring the inositol supplementation.

Moreover, the favorable results obtained by the PONTI study highlight the need of a more extensive evaluation on the role of myoinositol in preventing a greater number of NTDs, as compared to folic acid alone, or, alternatively, to study the effects of myoinositol on some subtypes of NTDs (i.e., folate-resistant NTDs).

However, even if a randomized clinical trial (RCT) would be necessary to demonstrate a statistically significant reduction in the rate of NTDs after myoinositol supplementation, it should be noted that, similar to rare diseases, the scarcity of at-risk subjects represents a barrier to overcome for the design of RCTs, which could only be performed with a greater/international recruitment effort. To overcome those difficulties, the authors suggest the possibility of a different approach (sequential study design), in which women at risk of NTDs undergo inositol supplementation after a previous affected pregnancy, in which folic acid alone was not effective. Prospectively, this approach could be seen as unethical and very long to be carried on. Alternatively, we propose that all the women with a previous NTD-affected pregnancy despite correct folic acid supplementation might take FA plus MI in the next pregnancy. As stated before, nonresponsiveness to FA supplementation should define a particular NTD subtype, namely, folate-resistant NTDs.

At our institution, over the course of 12 years, 27 pregnancies at high risk of folate-resistant NTDs were treated with 5 mg folic acid plus 1000 mg myoinositol supplementation in the periconceptional period, starting at least four weeks before pregnancy. All the mothers had undergone correct folic acid supplementation in their previous affected pregnancy/pregnancies. Despite the high recurrence risk, after combined folic acid and myoinositol supplementation, 29 babies not affected by NTDs were born. According to the different recurrence risks after one or more affected pregnancies, the expected frequency of NTDs in that selected population was 2–8 affected cases.

As no NTDs case was observed in this series, these results strengthen the PONTI results. Moreover, our data are consistent even with an Evidence-Based Medicine (EBM) approach. In fact, between the observed results and myoinositol plus folic acid periconceptional supplementation, a close temporal

relation and a strong relationship can be identified. Furthermore, given the biological mechanisms of inositol in neurogenesis, those results are clearly plausible, consistent, coherent, and specific [25]. Even if it seems difficult to combine the conclusions from a sequential study with those from a randomized clinical trial, both results strongly support a favorable effect of myoinositol supplementation in reducing the overall frequency of NTDs.

5. Conclusion

So, in light of the results reported before on the use of myoinositol in reducing the risk of recurrence of NTDs, we suggest that mothers with a previous NTD-affected pregnancy despite correct periconceptional folic acid intake should be considered at risk of folate-resistant NTDs and should be treated with myoinositol and folic acid in the next pregnancy.

In our experience, this approach has proved to be effective and without side effects on both the mothers and the fetuses for at least fifteen years.

In conclusion, after many years, attempts, and uncertainties, there are now many and consistent data that confirm the importance of myoinositol supplementation in the prevention of human NTDs, as well as the safety of that prophylactic approach. Moreover, given the lack of side effects associated with myoinositol periconceptional supplementation, we suggest that the administration of both folic acid and myoinositol should be further investigated even in normal pregnancies.

Competing Interests

The authors declare that they have no competing interest.

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Clinical Study

Different Effects of Myoinositol plus Folic Acid versus Combined Oral Treatment on Androgen Levels in PCOS Women

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Recently, myoinositol (myo-ins) and folic acid combination has gained an important role for treating Polycystic Ovary Syndrome (PCOS), in addition to combined oral contraceptives (COC). We aimed to examine myo-ins effects on anti-Mullerian hormone (AMH) levels and compare them with those ones obtained administering COC. In this prospective study, 137 PCOS patients, diagnosed according to Rotterdam criteria and admitted to the Reproductive Endocrinology and Infertility Outpatient Clinic at Dokuz Eylul University (Izmir, Turkey), were included. After randomization to COC ($n = 60$) and myo-ins ($n = 77$) arms, anthropometric measurements, blood pressure, Modified Ferriman Gallwey scores were calculated. Biochemical and hormonal analysis were performed, and LH/FSH and Apo B/A1 ratios were calculated. Data analysis was carried out in demographically and clinically matched 106 patients (COC = 54; myo-ins = 52). After 3-month treatment, increase in HDL and decreases in LH and LH/FSH ratio were statistically more significant only in COC group when compared with baseline (in both cases $p > 0.05$). In myo-ins group, fasting glucose, LDL, DHEAS, total cholesterol, and prolactin levels decreased significantly (for all $p < 0.05$). Progesterone and AMH levels, ovarian volume, ovarian antral follicle, and total antral follicle counts lessened significantly in both groups (for all $p < 0.05$). In PCOS treatment, MYO is observed more effective in reductions of total ovarian volume and AMH levels.

1. Introduction

Anti-Mullerian hormone (AMH), a polypeptide, secreted by the granulosa cells of the preantral and early developing antral follicles, has been shown to be a predictor of ovarian activity [1]. PCOS patients show an increased number of antral follicles; therefore they have higher circulating AMH levels than the healthy women [2]. The interactions between AMH and the hormonal profile characteristics in PCOS require a better assessment. The positive correlation between insulin resistance and serum AMH levels suggests that insulin exerts an action on AMH synthesis; however this aspect is not yet fully understood [3].

Polycystic Ovary Syndrome (PCOS) is a common endocrine disorder, affecting 6–10% of women in reproductive age. This syndrome is characterized by biochemical or clinical signs of hyperandrogenism, chronic anovulation, and polycystic ovaries [4]. It is frequently associated with insulin resistance and obesity. Evidence suggests that insulin resistance and its compensatory hyperinsulinemia play an important role in PCOS pathogenesis [5, 6]. Insulin is associated with hyperandrogenism; it acts synergistically with luteinizing hormone to increase the androgen production of theca cells [7]. Therefore, administration of insulin sensitizers ameliorates hyperandrogenemia and ovulatory functions [8].

Inositol (hexahydroxycyclohexane) belongs to the vitamin B complex group; it is a 6-carbon ring compound, having a hydroxyl group linked to each carbon of the ring, with nine possible stereoisomeric forms depending on the epimerization of the six hydroxyl groups. Among them, myoinositol

(myo-ins) is the mostly represented isoform, with very relevant biological functions [9]. Increasing evidence has demonstrated that myo-ins plays a key role in cell morphogenesis and cytogenesis, lipid synthesis, structure of cell membranes, and cell growth [10]. Myo-Ins administration improves hormonal profile, oocyte maturation, and insulin resistance; furthermore, it promotes the meiotic progression of germinal vesicle oocytes [11, 12]. Recent studies on PCOS patients showed a decrease of androgen levels and an improvement in ovulation and metabolic parameters after treatment with myo-ins and D-chiro-inositol (D-chiro-ins), which is another stereoisomeric form of inositol [13]. It was highlighted that very promising results were achieved administering myo-ins plus D-chiro-ins at their physiological range in plasma (i.e., 40 : 1) to ensure better clinical results in the PCOS therapy [13].

It is important to investigate the possibility of using AMH as a marker of some parameters: improved insulin resistance and decreased LH and androgen levels. This issue still needs further studies to reach a satisfactory framework [14].

Combined oral contraceptive (COC) pills, especially containing antiandrogen, are commonly used in the treatment of PCOS patients to suppress ovulation. In this study we focused our attention on the activity of myo-ins alone. Our primary outcome was to investigate the effect of myo-ins or COC on the clinical features, biochemical parameters, and AMH levels in PCOS patients. The secondary outcome of the study was to compare differences in changes after treatment with myo-ins or COC.

2. Methods

This is a randomized prospective trial carried out at the Department of Obstetrics and Gynecology (Dokuz Eylul University, Izmir, Turkey) between May 2013 and January 2014. This study started after the approval by the Clinical Research Ethics Committee of the Faculty of Medicine, Dokuz Eylul University, and the Turkish Health Ministry Drug and Medical Device Foundation. Informed written consent was obtained from all subjects. 137 patients diagnosed with Polycystic Ovary Syndrome and admitted to Dokuz Eylul University Faculty of Medicine, Department of Obstetrics and Gynecology, Division of Reproductive Endocrinology and Infertility Outpatient Clinic, were included in the study. The patients with odd numbers were allocated to COC group (n = 60), whereas the even ones to MYO group (n = 77) (Figure 1). The COC used was 2 mg cyproterone acetate and 0.035 mg ethinylestradiol (Diane 35; Schering AG, Istanbul, Turkey) daily. The COC was given for 21 days and in the following 7 days no drugs were given. This cycle was repeated for 3 months. In the myoinositol group, the product used contained 1 gram myoinositol and 100 μgram folic acid (INOFOLIC; Lo.Li. Pharma, Rome, Italy). The drug was used twice a day continuously for 12–16 weeks. In initial assessment, for patients who had regular menstrual cycle for both MYO and COC group, the blood samples for hormonal assessment except progesterone were conducted on day 2 or 3 of the menstrual cycle. The progesterone levels were analyzed on the 21st day of the menstrual cycle. The assessment after three months was done when the treatment finished and the patients had her first menstrual cycle after three months of treatment. The blood samples were again taken at day 2 or 3 of the cycle. The progesterone levels were analyzed on the 21st day of the cycle. For both groups the patients were not under medication during the assessment of the hormone levels. In oligo/amenorrhoeic patients, blood samples were collected after withdrawal bleeding induced by oral progestin (5 mg medroxyprogesterone acetate twice a day; Tarlusal; Deva Holding A.Ş., Istanbul, Turkey).

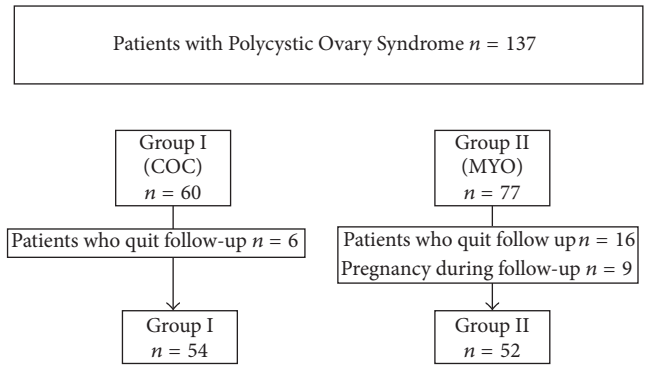

Figure 1: Flow chart of the study.

The diagnosis of PCOS was made according to the Rotterdam criteria; as prescribed, two out of three features were detected in the patients: oligomenorrhea (fewer than six menstrual periods in the preceding year) and/or anovulation; clinical and/or biochemical signs of hyperandrogenism; presence of ≥12 follicles in each ovary measuring 2–9 mm in diameter; and/or increased ovarian volume (>10 mL) [15]. Smoking, hyperprolactinemia, hypogonadotropic hypogonadism, pregnancy, thyroid disease, congenital adrenal hyperplasia, androgen-secreting tumors, and Cushing's syndrome were ruled out during the screening phase. None among the enrolled patients had taken, at least in the previous six months, oral contraceptives, antiandrogens, or any drug that could influence carbohydrate metabolism. Clinical evidence of hyperandrogenism was determined by the Ferriman Gallwey score ≥8 that reveals the presence of hirsutism and/or acne. Biochemical hyperandrogenism was defined as androgen level increase.

Initial physical examination included weight, height, and waist and hip circumferences, to calculate waist/hip ratio (WHR) and body mass index (BMI). BMI calculated as kg/m^2 was used as a measure of overall obesity. The WHR was used to assess the abdominal obesity. The waist circumference was measured at the midpoint of lowest margin of 12th rib and the lateral iliac crest during the normal expiration. The hip circumference was measured at the maximum distance between major trochanters. All anthropometric measurements were made by the same operator. Resting systolic and diastolic pressures were measured; after 2 minutes, the second measurement was performed and the mean values were determined.

Table 1: Comparison of demographic and clinical characteristics of group 1 and group 2.

	GROUP 1-COC (n = 54)	GROUP 2-MYO (n = 52)	$^{*}p$
Age (years)	22.79 ± 4.13	24.44 ± 4.78	0.061
Gravida	0.31 ± 0.88	0.30 ± 0.64	0.962
Parity	0.16 ± 0.46	0.11 ± 0.37	0.536
BMI (kg/m^2)	23.79 ± 4.24	25.33 ± 5.20	0.098
WHR	0.76 ± 0.15	0.83 ± 0.22	0.064
Mean artery pressure (mmHg)	82.16 ± 12.82	87.50 ± 10.31	**0.020**
Ferriman Gallwey score	12.53 ± 5.57	14.38 ± 6.41	0.116
Right ovary volume (cm^3)	9.50 ± 2.92	9.84 ± 2.75	0.545
Left ovary volume (cm^3)	8.93 ± 3.27	9.32 ± 2.42	0.495
Total ovary volume (cm^3)	18.44 ± 5.89	19.16 ± 4.58	0.486
Right ovary antral follicle count	16.09 ± 5.62	16.96 ± 6.38	0.458
Left ovary antral follicle count	14.81 ± 5.64	15.65 ± 5.71	0.449
Total antral follicle count	30.90 ± 10.27	32.61 ± 10.50	0.399

COC: combined oral contraceptives, MYO: myoinositol + folic acid, BMI: body mass index, and WHR: waist hip ratio. Independent t-test.

Serum levels of fasting plasma glucose and insulin, C-reactive protein (CRP), anti-Mullerian hormone (AMH), apoprotein B (Apo B), apoprotein A1 (Apo A1), high density lipoprotein (HDL) and low density lipoprotein (LDL), total cholesterol, triglyceride, follicle stimulating hormone (FSH), luteinizing hormone (LH), total and free testosterone, dehydroepiandrosterone sulfate (DHEAS), and sex hormone binding globulin (SHBG) were measured. Normal insulin sensitivity was defined by fasting serum glucose and insulin levels with homeostatic model of insulin resistance (HOMA-IR). HOMA-IR was calculated by the formula: HOMA-IR = fasting blood glucose (mg/dL) × fasting insulin (μIU/mL)/405.

Studies were performed within day 2 or 3 of the menstrual cycle. Fasting venous blood samples were taken between 08:00 am and 10:00 am after a 12-hour overnight fast. The blood samples were immediately centrifuged for 10 minutes and kept at −80°C in Eppendorf tubes until assayed.

Serum anti-Mullerian hormone (AMH) levels were analyzed with commercial kit according to manufacturer instructions based on the principle of competitive enzyme linked immunosorbent assay (ELISA) method (catalog number: CSB-E12756h, CUSABIO Biotech Co., USA). The microplate in the kit is precoated with goat-anti-rabbit antibody specific to AMH. Standard was reconstituted and prepared by serial dilution with sample diluent. Standards and undiluted samples are loaded into the appropriate microtiter plate wells with horseradish peroxidase (HRP) conjugated AMH and antibody specific to AMH. They were incubated for 60 minutes at 37°C. The competitive inhibition reaction was launched between HRP labeled AMH and unlabeled AMH with the antibody. Following a wash to remove any unbound substances, substrate A and B solutions were added and color develops in proportion to the amount of AMH. The color development is stopped and the intensity of the color is measured spectrophotometrically at a wavelength of 450 nm. A standard curve of known concentration (0, 0.375, 1.31, 4.69, 28.12, and 150 ng/mL) of AMH was established and the concentration of analyte in the samples was calculated accordingly. The ELISA assays of AMH had a sensitivity of 0.375 ng/mL; a detection range of 0.375–150 ng/mL; intra-assay coefficient of variation <%10, interassay coefficient of variation <%15, respectively.

Ovarian volume measurement was made by Medison Sono Ace X6 ultrasound system. For transvaginal ultrasonography, the probes used were EV4–9/10ED center frequency 6.5 mHz and ER4–9/10ED center frequency 6.5 mHz. For virgin patients transabdominal ultrasonography was used. The probe of the transabdominal ultrasonography was C2–8, center frequency 5 mHz. The presence of ≥12 follicles in each ovary measuring 2–9 mm in diameter was recorded. The total number of these follicles was accepted as follicle count. Longitudinal, transverse, and anteroposterior ovarian diameters were measured and multiplied by 0.5 to calculate the ovary volume.

Data were analyzed by using Statistical Program for Social Sciences (SPSS, version 16). The level of significance was accepted when $p < 0.05$. In Table 1, the test used was independent t-test. In Table 2, the test used was independent t-test. In Table 3, the test used was paired samples test. In Table 4, the test used was paired samples test.

3. Results

106 patients were analyzed, among them 54 patients were COC receivers (group 1), and 52 were myo-ins receivers (group 2). Baseline demographic, clinical characteristics, and ultrasound results of all patients are presented in Table 1. The mean age was 22.79 ± 4.13 years and 24.44 ± 4.78 years for groups 1 and 2, respectively. In group 1, 41 (75.9%) of 54 patients showed menstrual irregularity. At the end of the study period, patients in group 1 showed no menstrual irregularity, whereas the decrease of menstrual irregularity in group 2 was from 40 (76.9%) to 8 (15.4%) patients.

The baseline biochemical and hormonal values showed that patient groups were matched in these parameters except sex hormone binding globulin (SHBG) level which was higher in group 1 (Table 2).

Table 2: Comparison of baseline biochemical and hormonal parameters of patient groups.

	GROUP 1-COC ($n = 54$)	GROUP 2-MYO ($n = 52$)	$^{*}p$
Fasting glucose (mg/dL)	85.42 ± 8.60	86.48 ± 8.85	0.535
Fasting insulin (μIU/dL)	11.14 ± 8.87	12.06 ± 10.40	0.626
HOMA-IR	2.42 ± 2.08	2.62 ± 2.30	0.649
HDL (mg/dL)	51.14 ± 11.99	50.32 ± 12.50	0.731
LDL (mg/dL)	103.48 ± 31.53	112.84 ± 34.76	0.149
TG (mg/dL)	118.53 ± 67.18	119.44 ± 60.86	0.942
Total cholesterol (mg/dL)	180.51 ± 38.25	183.92 ± 35.55	0.636
CRP (mg/L)	2.54 ± 3.04	3.02 ± 3.27	0.437
Apo B/A1	0.62 ± 0.23	0.65 ± 0.24	0.537
DHEAS (μg/dL)	303.97 ± 138.57	318.29 ± 178.02	0.644
C-peptide (ng/mL)	2.84 ± 1.71	2.61 ± 1.41	0.461
Total testosterone (ng/dL)	1.10 ± 3.72	0.80 ± 0.47	0.573
Free testosterone (pg/mL)	2.01 ± 1.04	2.39 ± 1.72	0.176
Androstenedione (ng/mL)	4.25 ± 2.97	4.82 ± 3.79	0.397
17-OH progesterone (ng/mL)	1.22 ± 0.65	1.03 ± 0.58	0.119
SHBG (nmol/L)	59.40 ± 47.65	39.14 ± 29.54	**0.010**
Progesterone (ng/mL)	1.27 ± 1.88	1.37 ± 2.88	0.824
Estradiol (pg/mL)	63.98 ± 58.16	49.41 ± 26.42	0.102

COC: combined oral contraceptives; MYO: myoinositol + folic acid; HOMA-IR: Homeostatic Model Assessment-Insulin Resistance; HDL: high density lipoprotein; LDL: low density lipoprotein; TG: triglyceride; CRP: C-reactive protein; Apo B/A1: apoprotein B/A1; DHEAS: dehydroepiandrosterone sulfate; SHBG: sex hormone binding globulin; FSH: follicular stimulating hormone; LH: luteinizing hormone; TSH: thyroid stimulating hormone; AMH: anti-Mullerian hormone. Independent t-test.

Increase in HDL and decrease in LH and LH/FSH ratio were determined statistically significant only in group 1 when compared with the baseline values (for all $p > 0.05$) (Table 3). In group 2, the reduction of fasting glucose, LDL, DHEAS, total cholesterol, and prolactin levels was statistically significant (for all $p < 0.05$) (Table 3). Other parameters, such as total testosterone, DHEAS, and fasting glucose, were statistically improved in group 2, while no particular improvement was present in group 1 (Table 3). After 3-month treatment, AMH (for group 1 $p < 0.001$; for group 2 $p = 0.002$) levels as well as ovarian volumes, ovarian antral follicle count, and total antral follicle counts showed a statistically significant decrease in both groups (for all $p < 0.001$) (Table 3). When we evaluate progesterone levels at the end of the treatment, group 1 showed a statistically significant decrease whereas group 2 showed a statistically significant increase (for group 1 $p = 0.014$; for group 2 $p < 0.001$).

The lessening of ovarian volumes and AMH levels was statistically significantly more in the MYO group than COC group (for AMH; $p = 0.048$, for ovarian volumes $p = 0.040$) (Table 4).

4. Discussion

There are some therapies for Polycystic Ovary Syndrome, acting in different metabolic pathways. This prospective study observed the change in clinical, biochemical parameters and anti-Mullerian hormone levels after treatment with two different drug supplementations, myoinositol and combined oral contraceptives. Study data demonstrated that myo-ins regimen in PCOS patients positively affects metabolic parameters and modulates various hormonal factors deeply involved in the reproductive function and ovulation such as anti-Mullerian hormone.

It is well known that PCOS is characterized by hyperandrogenism and irregular menstrual cycles. Concerning menstrual irregularity, using myo-ins Papaleo et al. [16] and Gerli et al. [17] observed an improvement of 88% (after six months) and 70% (after 14 weeks). In the current study, 76.9% of patients in group 2, treated with myo-ins, had menstrual irregularity initially. Use of myo-ins was associated with a significant improvement, and menstrual irregularity was strongly reduced in the study population. This effect may be explained by the specific protein phosphorylation processes via protein kinase C, which modulates various cellular processes as a second messenger system.

Anti-Mullerian hormone is secreted primarily in the small antral follicles and AMH measurements correspond to granulosa cell activity, total antral follicle count, and ovarian volume. AMH has been also reported to be increased in PCOS women [18]. In addition, several studies showed that AMH might correlate with the severity of this syndrome [19]. In recent researches, positive correlations between AMH levels, ovarian volume, and total antral follicle count were demonstrated [20–22]. Several studies in the literature support that COC treatment decreases ovarian volume and antral follicle [23, 24]. Our analysis showed significant reduction in total antral follicle count and ovarian volume after treatment with COC and myo-ins (for both groups $p < 0.001$). In line with our study, the two researches of Genazzani demonstrated reduction in ovarian volume after myo-ins administration. However, they reported no changes in the

Table 3: Comparison of changes in all parameters after treatment between the groups.

	GROUP 1-COC (n = 54)			GROUP 2-MYO (n = 52)		
	Month 0	Month 3	$^*p$	Month 0	Month 3	$^*p$
BMI (kg/m^2)	23.79 ± 4.24	23.95 ± 4.28	0.166	25.33 ± 5.20	25.23 ± 5.02	0.656
WHR	0.76 ± 0.15	0.77 ± 0.16	0.175	0.83 ± 0.22	0.82 ± 0.21	0.230
Ferriman Gallwey score	12.53 ± 5.57	12.09 ± 5.40	**<0.001**	14.38 ± 6.41	14.40 ± 6.31	0.859
Fasting glucose (mg/dL)	85.42 ± 8.60	85.40 ± 9.25	0.990	86.48 ± 8.85	82.55 ± 14.03	**0.028**
Fasting insulin (μIU/dL)	11.14 ± 8.87	10.57 ± 8.81	0.521	12.06 ± 10.40	10.93 ± 12.57	0.531
HOMA-IR	2.42 ± 2.08	2.33 ± 2.09	0.633	2.62 ± 2.30	2.36 ± 2.97	0.527
HDL (mg/dL)	51.14 ± 11.99	54.50 ± 12.15	**0.016**	50.32 ± 12.50	50.13 ± 10.88	0.870
LDL (mg/dL)	103.48 ± 31.53	99.12 ± 27.51	0.102	112.84 ± 34.76	105.55 ± 29.15	**0.031**
TG (mg/dL)	118.53 ± 67.18	123.20 ± 60.87	0.554	119.44 ± 60.86	117.00 ± 69.45	0.703
Total cholesterol (mg/dL)	180.51 ± 38.25	179.74 ± 34.01	0.822	183.92 ± 35.55	179.63 ± 28.18	0.200
DHEAS (μg/dL)	303.97 ± 138.57	295.22 ± 116.51	0.492	318.29 ± 178.02	284.16 ± 136.73	**0.043**
C-peptide (ng/mL)	2.84 ± 1.71	2.75 ± 2.05	0.770	2.61 ± 1.41	2.36 ± 1.46	0.220
Total testosterone (ng/dL)	1.10 ± 3.72	1.30 ± 5.49	0.826	0.80 ± 0.47	0.54 ± 0.22	**<0.001**
Free testosterone (pg/mL)	2.01 ± 1.04	1.82 ± 0.83	0.153	2.39 ± 1.72	2.22 ± 0.84	0.460
Androstenedione (ng/mL)	4.25 ± 2.97	3.74 ± 1.95	0.098	4.82 ± 3.79	4.75 ± 3.10	0.870
SHBG (nmol/L)	59.40 ± 47.65	74.16 ± 53.78	**0.030**	39.14 ± 29.54	42.22 ± 25.44	0.479
Progesterone (ng/mL)	1.27 ± 1.88	0.70 ± 0.59	**0.014**	1.37 ± 2.88	4.41 ± 4.35	**<0.001**
Estradiol (pg/mL)	63.98 ± 58.16	46.59 ± 32.20	0.051	49.41 ± 26.42	47.03 ± 25.04	0.635
FSH (mIU/mL)	5.20 ± 2.04	5.59 ± 1.77	0.246	5.46 ± 3.78	5.89 ± 1.61	0.397
LH (mIU/mL)	7.61 ± 4.19	4.43 ± 2.85	**<0.001**	8.12 ± 5.17	8.52 ± 6.57	0.680
LH/FSH ratio	1.51 ± 0.70	0.81 ± 0.47	**<0.001**	1.66 ± 0.94	1.45 ± 0.87	0.146
Prolactin (ng/mL)	12.88 ± 7.19	13.66 ± 4.67	0.370	13.33 ± 6.04	10.81 ± 5.00	**0.001**
TSH (μU/mL)	1.70 ± 0.83	1.79 ± 0.82	0.481	1.65 ± 0.71	1.83 ± 0.77	0.124
AMH (ng/mL)	9.39 ± 6.60	8.51 ± 6.20	**<0.001**	11.51 ± 11.50	9.07 ± 9.32	**0.002**
Right ovary volume (cm^3)	9.50 ± 2.92	8.12 ± 2.70	**<0.001**	9.84 ± 2.75	7.81 ± 2.43	**<0.001**
Left ovary volume (cm^3)	8.93 ± 3.27	7.94 ± 3.03	**<0.001**	9.32 ± 2.42	7.38 ± 1.69	**<0.001**
Total ovary volume (cm^3)	18.44 ± 5.89	16.06 ± 5.51	**<0.001**	19.16 ± 4.58	15.19 ± 3.56	**<0.001**
Right ovary follicle count	16.09 ± 5.62	11.05 ± 5.65	**<0.001**	12.96 ± 6.38	10.82 ± 6.02	**<0.001**
Left ovary follicle count	14.81 ± 5.64	10.75 ± 5.97	**<0.001**	15.65 ± 5.71	11.05 ± 5.95	**<0.001**
Total antral follicle count	30.94 ± 10.27	21.81 ± 10.99	**<0.001**	32.61 ± 10.50	21.88 ± 11.23	**<0.001**

COC: combined oral contraceptives; MYO: myoinositol + folic acid; BMI: body mass index; WHR: waist hip ratio; HOMA-IR: Homeostatic Model Assessment-Insulin Resistance; HDL: high density lipoprotein; LDL: low density lipoprotein; TG: triglyceride; DHEAS: dehydroepiandrosterone sulfate; SHBG: sex hormone binding globulin; FSH: follicular stimulating hormone; LH: luteinizing hormone; TSH: thyroid stimulating hormone; AMH: anti-Mullerian hormone. Paired samples test.

Table 4: Change in AMH levels, ovary volumes, and total antral follicle counts between group 1 and group 2.

	Δ_{COC} (n = 54)	Δ_{MYO} (n = 52)	$^*p$
AMH (ng/mL)	0.88 ± 1.72	2.44 ± 5.45	**0.048**
Total ovary volume (cm^3)	2.37 ± 2.81	3.97 ± 4.84	**0.040**
Total antral follicle count	9.09 ± 6.77	10.73 ± 10.98	0.356

COC: combined oral contraceptives: MYO: myoinositol + folic acid; AMH: anti-Mullerian hormone. Δ indicates change. Paired samples test.

total antral follicle count after myo-ins treatment [25, 26]. The predictability of a successful therapy can be monitorized by changes in AMH levels. Although there are no previously published data about changes in serum AMH levels during myo-ins therapy, there are several studies that observed AMH changes after treatment with COC [27–29]. Li et al. examined 95 women in five different groups and they found no significant change in AMH levels before and after treatment with combined oral contraceptives [27]. In contrast, decrease in AMH levels after COC was shown in previous studies and it has been confirmed in our study [28, 29]. The decline in AMH levels may be related to the suppression of ovarian function with COC. Research has been mainly focused on COC's activity and there are no clinical studies on myo-ins effect on AMH levels. Genazzani et al. reported that myo-ins supplementation, in PCOS patients, affected metabolic parameters

such as insulin sensitivity and modulated positively hormonal factors like LH, FSH, and testosterone [25]. There was one retrospective study which showed decreased AMH levels after D-chiro-ins [30]. To our knowledge, the present study is the first one in the scientific literature indicating statistically significant decrease in AMH levels after 12–16 weeks of myo-ins use. We believe that suppression in AMH levels may be explained by the reduction of total antral follicle count and ovarian volume. However, the mechanism between decrease in total antral follicle count and myo-ins therapy is not clearly understood yet. We hypothesize that whereas hyperinsulinemia may stimulate the development of antral follicles and recover the sensitivity of granulosa cells to FSH, therefore leading to increase in the number of follicles, ovarian volume, and AMH levels, myo-ins may improve clinical and hormonal features of PCOS patients by enhancing insulin sensitivity that decreases hyperinsulinemia.

In our trial, we compared the ovarian volumes, total antral follicle count, and serum AMH levels after treatment with COC and myo-ins. Patients in the study group showed significantly more relevant reduction in AMH levels ($p = 0.048$) and ovarian volume ($p = 0.040$) than the COC group. The decrease in total antral follicle count for myo-ins and COC group was similar ($p = 0.356$). Based on our data, we believe that myo-ins is more effective compared to COC in lowering AMH levels and ovarian volumes. Additionally myo-ins has much less side effects and is without contraindications. Myoinositol cannot replace COC for contraception and may be preferred for patients with infertility who desire to conceive pregnancy. Also, myoinositol can be an option in patients with hirsutism, oligoanovulation, and symptoms of hyperandrogenism who have a contraindication to use COC. Further studies are needed to compare myoinositol and antiandrogenic drugs. Myoinositol is not a primary treatment; it is an adjunct therapy to improve insulin resistance.

In the present study, we also observed the effect of COC and myo-ins on fasting insulin, fasting glucose, and HOMA-IR. The results showed no significant change in insulin or HOMA-IR in both groups. Only PCOS patients, who received myo-ins, presented a significant reduction in glucose levels. However, we know that our study had a too short duration to be capable of detecting myo-ins effect on insulin and HOMA-IR. In contrast with our data, Zacchè et al. demonstrated that fasting insulin and HOMA-IR values were decreased after myo-ins treatment [31]. Villaseca et al. reported that COC did not affect fasting insulin or fasting glucose levels or HOMA-IR [32]. In Minozzi et al.'s study, patients were divided into two groups as COC + myo-ins receivers and only COC receivers. The results showed a significant reduction in glucose levels only in COC + myo-ins group, whereas no significant change was observed in COC group. The results of Minozzi et al.'s prospective study showed that the combination of COC and myo-ins was a more effective treatment for clinical symptoms of PCOS and in controlling endocrine disorders and insulin resistance [33].

Many studies have been carried out in PCOS patients with high serum androgen levels to determine the clinical implications. Most of the agents used in the treatment aimed to reduce serum androgen levels. The results of this study showed that myoinositol treatment was able to improve some key parameters such as the total testosterone level. The reduction of testosterone is a key outcome in the management of PCOS women, because of the typical symptoms related to the hyperandrogenic status, affecting the PCOS pathway. These results could be due to the insulin sensitizing action of myoinositol and the sequent downregulation of the androgens production at ovarian level [31]. The distinct effect of myoinositol on testosterone, AMH, and ovarian volume is surprising and this could be explained by a reduction in hyperinsulinism. In our study, we did not observe significant reduction in insulin and HOMA-IR, but still there is a decrease in HOMA-IR blood levels. To observe the significant change, the study period should be extended. Also, the number of the patients in the study should be raised.

Zacchè et al. demonstrated a decrease in free testosterone and total testosterone after myo-ins treatment, but no significant change in androstenedione levels [31]. When we evaluate the changes in androgen levels in our study, we have seen that myo-ins treatment reduced DHEA-S and total testosterone levels, but no significant differences in SHBG and androstenedione were found. In the present study, whereas COC group showed a significant increase in SHBG levels, statistical significance was not observed in the increase of DHEAS, free testosterone, and androstenedione and in the reduction in total testosterone levels. Literature data indicate that if the treatment duration was extended, changes in androgen levels might become significant. Therefore, it is more appropriate planning a long term therapy in the management of androgen excess, anovulation, and insulin resistance.

5. Conclusion

This study shows that the combination of myoinositol plus folic acid should be considered in the treatment of PCOS patients. Considering previous studies, myo-ins has also reduced hirsutism, yet more slowly than COC. Studies in the literature indicate that myoinositol exerts positive effects on insulin resistance, but further researchers are still required to clarify the mechanism. The present study is the first one reporting that myoinositol is superior to COC in terms of lowering androgens levels. Also, myoinositol effect on ovarian volume and AMH levels is remarkable when compared to COC.

Competing Interests

There is no conflict of interests that could be perceived as prejudicing the impartiality of the research reported.

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Clinical Study

Myoinositol and D-Chiro Inositol in Improving Insulin Resistance in Obese Male Children: Preliminary Data

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Myoinositol and D-chiro inositol, which are inositol isomers, have been shown to possess insulin-mimetic properties and to improve insulin resistance, especially in women with polycystic ovary syndrome. However, it has not been determined if this relationship exists also in children. Based on these previous findings, we hypothesized that inositol could be effective in improving insulin sensitivity in children with insulin resistance. To evaluate this hypothesis, we administered both inositol formulations before carrying out an oral glucose tolerance test (OGTT) in a group of obese insulin-resistant male children with high basal insulin levels and compared the values obtained with an OGTT previously conducted without inositol, in the same group, with unchanged BMI. Our results confirm that myoinositol and D-chiro inositol acutely reduce insulin increase after glucose intake mainly in children with high basal insulin level.

1. Introduction

Myoinositol (MI) and D-chiro inositol (DCI) are isomeric forms of inositol that were found to have insulin-like properties, acting as second messengers in the insulin intracellular pathway; both of these molecules are involved in the increasing insulin sensitivity of different tissues to improve metabolic and ovulatory functions [1–3].

Myoinositol is the predominant form that can be found in nature and food. It is produced by the human body from D-glucose, but it is present in all living cells as membrane phospholipids and phytic acid. In food it is contained especially in pulses (beans, grains, and nuts) and fruits (in particular citrus fruits).

DCI and MI have different physiological roles since the former is crucial for glycogen synthesis while the latter increases cellular glucose uptake [4]. Each tissue has its own MI/DCI ratio, which is maintained through the conversion of myoinositol to D-chiro inositol occurring in tissues expressing the specific epimerase. High DCI levels are present in glycogen storage tissues, such as fat, liver, and muscle, whereas very low levels of DCI are typical of tissues with high glucose utilization, such as the brain and heart [5, 6].

The inositol is mainly catabolized by the kidney, which seems to be an important regulator of plasma inositol concentrations; however urinary excretion represents only a small fraction.

Indeed, deficiency or abnormalities in inositol metabolism induce a defect in glucose uptake and have been linked to insulin resistance and long term microvascular complications of diabetes [7].

A depletion of intracellular myoinositol and an excessive urinary excretion, along with lower level of chiro inositol and lower excretion, have been frequently observed in type II diabetic patients and in other conditions associated with insulin resistance, such as polycystic ovary syndrome, gestational diabetes, and metabolic syndrome. In general, certain data suggest that chiro inositol deficiency or imbalance is related more directly to insulin resistance itself, rather than to type II diabetes [4].

The chiro inositol deficiency observed in urine has also been confirmed in muscle and in blood, indicating a general

Table 1: Characteristics of enrolled subjects at baseline.

Variable	N	Mean ± SD	Median [25th–75th]
Age (years)	23	11.5 ± 2.3	12.0 [10.0–13.0]
Weight (kg)	23	70.4 ± 14.9	75.5 [56.5–82.7]
Height (cm)	23	153.0 ± 13.3	155.0 [143.0–163.0]
BMI (kg/m^2)	23	29.8 ± 3.1	30.1 [27.6–31.4]
Fasting glucose (mg/dL)	23	86.7 ± 4.5	87.0 [84.0–89.0]
Fasting insulin (μU/mL)	23	16.6 ± 9.2	14.0 [11.6–18.8]

defect in the body. This finding has provided the basis for initial trials of the administration of D-chiro inositol in STZ diabetic rats, monkeys, and subsequently humans [2, 8].

Thus administration of myoinositol and D-chiro inositol might play a role in restoring better insulin sensitivity, as well as delaying the onset of diabetes complications.

In this preliminary study obese male children have undergone an OGTT without inositol and then in association with inositol, in order to find out if the acute administration of inositol has a positive effect on insulin sensitivity.

We hypothesized that there may be a direct relation between higher BMI and a greater increase of insulin after glucose administration and that the effect of inositol might be different in subjects with higher BMI. We also proposed the hypothesis that there might be a relation between fasting insulin level and degree of insulin increase after glucose intake.

2. Materials and Methods

Twenty-three consecutive obese patients, aged 11.5 ± 2.3 (range 7–15), with a mean BMI of 29.8 ± 3.1 kg/m^2 (mean ± SD) were recruited. Obesity was normalized according to Cacciari graphic percentiles [9].

Exclusion criteria were delayed puberty, hypogonadism (such as Klinefelter syndrome), thyroid dysfunctions, and obesity-linked genetic disease.

Anthropometric measures, like waist-hip ratio and percent of body fat, blood pressure, and blood samples for determination of total cholesterol, LDL, HDL, and triglycerides, were assessed. Blood samples for glucose and insulin were collected before and after the OGTT. Baseline patient characteristics are shown in Table 1.

All patients consumed a normocaloric diet, balanced for macronutrient distribution, in accordance with the national guidelines for the treatment of childhood obesity [10, 11]. Specifically, it is recommended that children follow, for a 6-month period, a normocaloric diet (daily caloric intake by age and sex [12]) consisting of protein (12%–15%), carbohydrates (55%–60%), fat (25%–30%; <10% saturated fatty acids, polyunsaturated fatty acids up to 10%, and monounsaturated fatty acids up to 15%), and fiber (range: age (year) plus 5 g-age (year) plus 10) [11, 12].

Additionally, it was recommended that children engage in at least 60 min of moderate-to vigorous-intensity physical activity daily, based on walking and tailored to individual preference [13].

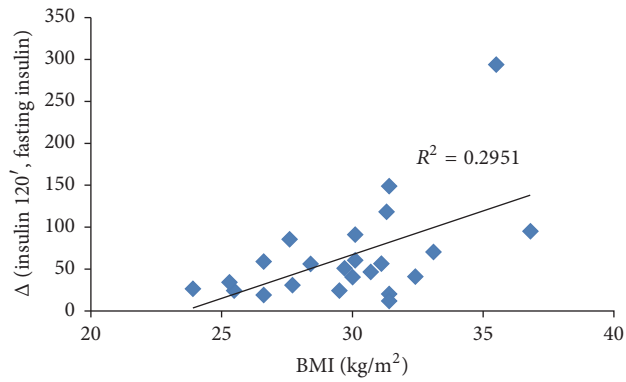

Figure 1: Correlation between BMI and insulin increase from fasting to 120 minutes after OGTT without inositol in the whole sample. $p = 0.0336$.

After nutritional intervention patients who recorded in the first OGTT fasting insulin ≥15 μU/mL and thus are considered as insulin-resistant were submitted to a second OGTT, which was preceded by the administration of 1 soft gel capsule of inositol [myoinositol 1100 mg + D-Chiro inositol 27.6 mg + folic acid 400 μg; Inofolic Combi®, Lo.Li Pharma S.r.l., 00156 Rome, Italy]. In these insulin-resistant patients, blood samples for glucose and insulin were collected before and after the OGTT and data from the first and second OGTT were compared.

3. Statistical Analysis

To assess the differences between variables over time we performed the Wilcoxon signed rank sum test assuming $p \leq 0.05$ as significant level. The Spearman Correlation Coefficient was employed to identify a correlation between BMI and insulin increase after OGTT and between fasting insulin and insulin level after glucose load (Figures 1, 2, and 3). Correlation is used to assess the strength and direction of the linear relationship between two variables. It measures the association, not the casual relationship [14].

4. Results and Discussion

4.1. Results. Figure 1 shows that the baseline correlation between BMI and insulin increase in the whole sample was statistically significant ($R^2 = 0.2951$; $p = 0.0336$), confirming that higher BMI is associated with a greater insulin increase.

Therefore, we performed an OGTT preceded by inositol administration in a subgroup of 11 children only, where the high fasting insulin level (≥15 μU/mL) suggested increasing insulin resistance.

Figure 2 shows the correlation between basal insulin value and its increase after the OGTT performed without inositol and with inositol in the patients with basal insulin ≥15 μU/mL. While correlation was strong and statistically significant in the first case ($R^2 = 0.2281$; $p = 0.0009$), it lost significance when the OGTT was preceded by inositol administration ($R^2 = 0.2447$; $p = 0.0767$). In this case, basal

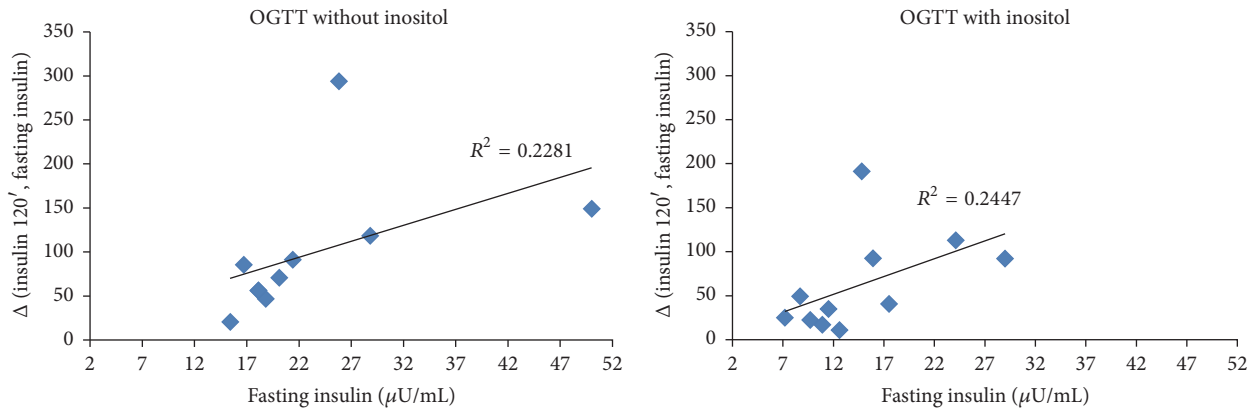

Figure 2: Correlation between basal insulin level and insulin increase from fasting to 120 minutes after OGTT without or with inositol in subjects with a basal insulin level ≥15 μU/mL. (a) $p = 0.0009$. (b) $p = 0.0767$.

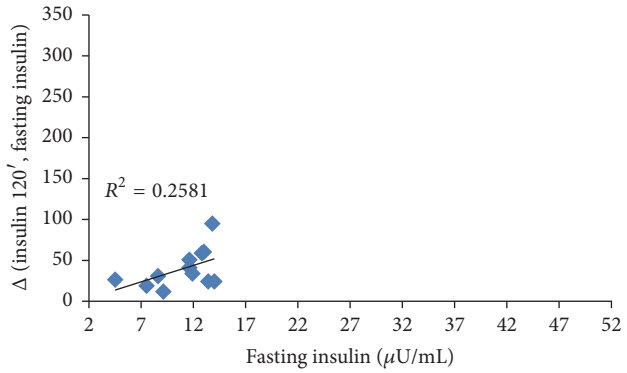

Figure 3: Correlation between basal insulin level and insulin increase from fasting to 120 minutes after OGTT without inositol in subjects with a basal insulin level <15 μU/mL. $p = 0.2198$.

insulin level and insulin increase got close to those observed in the children who had a basal insulin level ≤15 μU/mL (Figure 3), suggesting that inositol is more effective in lowering insulin increase in children with higher basal insulin level, though this assumption is still uncertain since only a few samples have been tested.

Table 2 reports baseline values and variations of blood glucose and insulin after 120 minutes in the two groups of children, respectively, with insulin level ≥ or ≤15 μU/mL. Both groups recorded a significant increase of glucose and insulin after 120 minutes: in particular, in the first group the mean glucose increase after the OGTT was 29.4 ± 17.9 mg/dL ($p = 0.001$) and mean insulin increase was 93.5 ± 75.9 μU/mL ($p = 0.001$); in the second one mean glucose increase after the OGTT was 12.3 ± 15.0 ($p = 0.0029$) and mean insulin increase was 39.8 ± 23.3 ($p = 0.0005$).

Table 3 shows the comparison between data without inositol and the OGTT performed after inositol administration in the patients with baseline insulin ≥15 μU/mL: fasting glucose was not significantly different from the previous test (88.5 ± 3.4 versus 86.9 ± 3.9 mg/dL) and there was a small nonsignificant reduction of glucose after 120 minutes (117.9 ± 19.2 versus 106.2 ± 18.0 mg/dL), while a reduction of fasting insulin (22.6 ± 10.0 versus 14.7 ± 6.7 μU/mL; $p = 0.001$) was noted, and a decrease of insulin after 120 minutes (116.1 ± 81.4 versus 77.3 ± 58.4 μU/mL; $p = 0.0176$). In addition, in these 11 patients mean BMI after six months was not significantly different from the baseline (31.0 ± 2.1 versus 31.0 ± 2.6 kg/m^2). In fact, they resulted to have had a BMI variation ≤2.5 kg/m^2. In particular, some children have obtained a BMI reduction ($n = 4$), stabilization ($n = 3$), or an increase ($n = 4$), in a similar distribution. Thus BMI variation did not influence insulin variations and the results can be attributed to inositol only.

4.2. Discussion. The effectiveness of inositol in lowering plasma glucose concentration and postprandial blood glucose levels has been reported in several cases of mellitus diabetes in rats, monkeys, and humans. These studies showed that this effect was related to insulin-sensitizing activity [15–18]. D-Pinitol exerted an acute and chronic insulin-like antihyperglycaemic effect on glucose transport in STZ-diabetic mice, but acute administration of D-pinitol did not significantly alter plasma glucose or insulin concentration over 6 hours in severely insulin-resistant ob/ob mice [19]. When administered to insulin-resistant and diabetic monkeys, D-chiro inositol accelerated glucose disposal and activated glycogen synthase in muscle biopsies beyond that of maximal insulin stimulation [20]. Pinitol was administered to humans with type II diabetes for 13 weeks and it significantly decreased mean fasting plasma glucose and insulin and improved lipid profile, while four weeks of pinitol treatment did not alter insulin-mediated glucose disposal in individuals with obesity and mild type II diabetes [21, 22].

It seems that body weight is not significantly affected by inositol treatment [19, 21] while inositol has been reported to improve insulin sensitivity and ovulatory function in young women affected by polycystic ovary syndrome. When the effect of inositol has been investigated in postmenopausal women with metabolic syndrome and in pregnant women with gestational diabetes, it resulted in improvements of fasting serum insulin and blood glucose levels [23–25]. In particular, in pregnant women with a family history of

Table 2: Baseline values and changes by baseline insulin level.

		Basal insulin level ≥15 μU/mL			Basal insulin level ≤15 μU/mL			
Variable	N	Mean ± SD	Median [25th–75th]	p^*	N	Mean ± SD	Median [25th–75th]	p^*
Fasting glucose (mg/dL)	11	88.5 ± 3.4	87.0 [86.0–90.0]		12	85.0 ± 4.9	85.0 [82.0–88.0]	
Blood glucose 120′ (mg/dL)	11	117.9 ± 19.2	116.0 [98.0–136.0]		12	97.3 ± 16.2	94.0 [87.0–99.5]	
Δ (blood glucose 120′, fasting glucose)	11	29.4 ± 17.9	27.0 [14.0–46.0]	0.001	12	12.3 ± 15.0	6.5 [3.0–17.5]	0.003
Fasting insulin (μU/mL)	11	22.6 ± 10.0	18.8 [16.7–25.8]		12	11.0 ± 2.9	11.8 [8.9–13.2]	
Insulin 120′ (μU/mL)	11	116.1 ± 81.4	90.6 [65.5–147.0]		12	50.7 ± 24.9	42.7 [34.3–67.1]	
Δ (insulin 120′, fasting insulin)	11	93.5 ± 75.9	70.5 [46.7–118.2]	0.001	12	39.8 ± 23.3	32.5 [24.2–54.9]	0.000

*Wilcoxon signed rank sum test.

Table 3: Values at baseline (OGTT without inositol) and after 6 months (OGTT with inositol) of the subjects with a basal insulin level ≥15 μU/mL.

	OGTT without inositol		OGTT with inositol		
Variable	Mean ± SD	Median [25th–75th]	Mean ± SD	Median [25th–27th]	p^*
BMI (kg/m^2)	31.0 ± 2.1	31.1 [30.0–31.4]	31.0 ± 2.6	31.4 [29.8–32.5]	0.867
Weight (kg)	72.1 ± 15.7	75.9 [56.3–83.4]	74.4 ± 16.0	78.9 [60.0–84.8]	0.201
Fasting glucose (mg/dL)	88.5 ± 3.4	87.0 [86.0–90.0]	86.9 ± 3.9	87.0 [83.0–90.0]	0.177
Blood glucose 120′ (mg/dL)	117.9 ± 19.2	116.0 [98.0–136.0]	106.2 ± 18.0	106.0 [93.0–113.0]	0.101
Fasting insulin (μU/mL)	22.6 ± 10.0	18.8 [16.7–25.8]	14.7 ± 6.7	12.6 [9.7–17.5]	0.001
Insulin 120′ (μU/mL)	116.1 ± 81.4	90.6 [65.5–147.0]	77.3 ± 58.4	57.9 [32.0–121.0]	0.017

*Wilcoxon signed rank sum test.

type 2 diabetes a supplement of myoinositol throughout the pregnancy also reduced the 1-hour glycemia at the OGTT stage and reduced the incidence of gestational diabetes [26].

However, those studies only include women with a specific hormonal status (gestation, menopause, or PCOS) which does not allow us to confirm a similar effect of inositol supplementation in other contexts; therefore the true mechanism of action is still unclear.

To our knowledge this is the first study to test the effect of inositol in children, even though our results are only preliminary.

The main aim of this process was to determine whether an acute effect of inositol on insulin sensitivity in children exists, when administered before a glucose tolerance test.

Our results indicate that insulin increase after glucose intake may be attenuated when inositol is administered before the OGTT. In particular, it seems that inositol reduces insulin increase mainly in children with high basal insulin level. So patients with high fasting insulin might be the ones to benefit more from the administration of inositol.

In our study the observed correlations are not high, but this may be due to the low number of units in the sample analyzed. They seem however indicative of an association between the observed insulin increase and the level of basal insulin that should be analyzed in detail with ad hoc larger studies.

5. Conclusions

Myoinositol and D-chiro inositol have been shown to reduce insulin increase after glucose intake in obese children. Further investigations and larger studies are required to define the physiological and potential therapeutic effects of their administration, but they could be effective oral nonpharmacological agents in the prevention of type II diabetes in children.

Ethical Approval

The pill employed is not a drug but contains integrative substances. The present observational study is not experimental research and thus does not require ethical committee approval.

Competing Interests

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

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Review Article

Effects of Inositol(s) in Women with PCOS: A Systematic Review of Randomized Controlled Trials

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Polycystic ovary syndrome (PCOS) is a common endocrine disorder, with complex etiology and pathophysiology, which remains poorly understood. It affects about 5–10% of women of reproductive age who typically suffer from obesity, hyperandrogenism, ovarian dysfunction, and menstrual irregularity. Indeed, PCOS is the most common cause of anovulatory infertility in industrialized nations, and it is associated with insulin resistance, type 2 diabetes mellitus, and increased cardiovascular risk. Although insulin resistance is not included as a criterion for diagnosis, it is a critical pathological condition of PCOS. The purpose of this systematic review is the analysis of recent randomized clinical trials of inositol(s) in PCOS, in particular myo- and D-chiro-inositol, in order to better elucidate their physiological involvement in PCOS and potential therapeutic use, alone and in conjunction with assisted reproductive technologies, in the clinical treatment of women with PCOS.

1. Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders affecting women of reproductive age. PCOS is associated with a wide range of maladies, such as hormonal and metabolic impairments, ovarian dysfunction, and menstrual irregularity. According to the Rotterdam criteria developed in 2003, PCOS is diagnosed if two out of the three following features are met: chronic oligo- or anovulation, anatomically polycystic ovaries on ultrasonography, and clinical and/or biochemical hyperandrogenism [1]. Although not included as criteria, insulin resistance and hyperinsulinemia are important etiologic factors associated with the typical clinical signs and hormonal disorders of PCOS. Indeed, insulin resistance along with hyperinsulinemia affects approximately 40–50% of PCOS patients, both lean and obese [2–6]; however, in obese women with PCOS the prevalence of insulin resistance accompanied by compensatory hyperinsulinemia approaches 80% [7]. Treatment of PCOS with insulin-sensitizing drugs, such as metformin, troglitazone, and pioglitazone, has been shown to improve ovulatory function and reduce circulating androgens, corroborating the critical link between insulin resistance and the pathogenesis of this syndrome. Of these insulin-sensitizing agents, metformin is most commonly used in the treatment of PCOS, although it has no official indication outside of type 2 diabetes in many countries and therefore it is considered as an off-label product when used in nondiabetic women with PCOS. Nevertheless, nausea, diarrhea, and weight increase are side effects of metformin, which reduce patients' compliance and the suitability of its use [3, 8, 9].

In the past two decades, several studies have reported the effectiveness of inositol(s), mainly the two stereoisomers myo-inositol (Myo-Ins) and D-chiro-inositol (D-chiro-Ins), in improving the pathological conditions associated with PCOS [3, 8–14]. Indeed, Myo-Ins and D-chiro-Ins have been shown to play different roles in the physiology and treatment of PCOS [15]. In the ovary, D-chiro-Ins is involved

in insulin-mediated androgen synthesis [16], whereas Myo-Ins mediates glucose uptake and follicle stimulating hormone (FSH) signaling [14, 15, 17, 18]. In human ovaries, 99% of the intracellular pool of inositol consists of Myo-Ins and the remaining part consists of D-chiro-Ins [17]; imbalance of ovarian Myo-Ins and D-chiro-Ins concentrations, like a putative Myo-Ins deficiency, might impair the FSH signaling, as observed in PCOS patients [17–19]. D-chiro-Ins is synthetized from Myo-Ins through the epimerase enzyme, which in turn is stimulated by insulin [19]. The epimerase activity is increased in the theca cells, causing a deficit of Myo-Ins [19] and this appears to be a critical factor in the pathogenesis of PCOS. Indeed, reduced intraovarian Myo-Ins may adversely affect glucose uptake and metabolism of both oocytes and follicular cells. Since oocytes are characterized by high glucose consumption this would compromise oocyte quality.

Several studies have emphasized the pivotal role of Myo-Ins in improving oocyte quality [10, 14, 25, 31, 32]. Myo-Ins and D-chiro-Ins are intracellularly incorporated into inositol phosphoglycans (IPGs), which are second messengers of insulin, and some actions of insulin are mediated by these IPG mediators. A number of studies have suggested that insulin pathway impairment could be due to dysregulation of the IPG second messenger system [33, 34]. This is consonant with the studies of Nestler et al. which suggest that altered metabolism of inositol or IPG mediators contribute to the insulin resistance of women with PCOS [13]. Indeed, they have demonstrated that D-chiro-Ins supplementation replenished stores of the mediator and improved insulin sensitivity in both lean and obese women with PCOS [12, 13].

Given the physiologic role of inositol(s) in oocyte and spermatozoa development, the 2013 Florence International Consensus Conference on myo- and D-chiro-inositol in obstetrics and gynecology addressed the use of inositol(s) in assisted reproductive technologies (ART) [35]. In addition, a previous systematic review by Unfer et al. provided an overview of the clinical outcomes of Myo-Ins as a treatment to improve ovarian function, as well as hormonal and metabolic parameters, in PCOS women [14]. In the present systematic review, we present updated information about inositol(s), in particular Myo-Ins and D-chiro-Ins, through an analysis of recently published reports, in order to better outline the physiological involvement and clinical use of inositol(s) in PCOS and ART.

2. Methods

A critical review of the literature was performed by searching core databases to select pertinent scientific articles: Medline, Amed, and the Cochrane Library. We conducted a search over the period from January 1999 to May 2016, and only randomized controlled trials (RCTs), involving women with PCOS, were included in the present study. Search terms included "inositol," "myo-inositol," "D-chiro-inositol," "polycystic ovary syndrome," "oocyte quality," "ovarian stimulation," "in vitro fertilization," "ovarian function," and "insulin resistance." No language restrictions were imposed. Data from treatments with Myo-Ins or D-chiro-Ins in combination with other drugs, as well as animal and in vitro investigations, were excluded. Full articles were obtained through either our own library or interlibrary loan, for all published studies that were considered eligible for inclusion in the review. As described below, a total of 12 studies were finally included for review.

The main outcomes we aimed to focus on were the following: glucose and insulin sensitivity, 17β-estradiol (E2), testosterone (T), androstenedione (A), the homeostatic model assessment (HOMA) index, sex hormone binding globulin (SHGB), r-FSH, stimulation days, oocyte quality, embryo quality, biochemical pregnancies, and pregnancy rate.

3. Results of the Literature Search

The systematic search yielded 102 papers for consideration. A total of 69 studies were excluded during the screening phase as not being pertinent. Of the remaining 33 studies, 21 did not meet the selection criteria. This left 12 studies that were included and analyzed in the final review (Tables 1, 2, 3, and 4). All the RCTs analyzed in this review studied patients with PCOS.

Eight trials evaluated the effect of Myo-Ins administration on hormonal levels and oocyte quality [10, 11, 20, 21, 25–27, 29]. In one trial, the effects of different concentrations of D-chiro-Ins on the oocytes quality were assessed [28]. Three RCTs evaluated the effects of combined therapy with Myo-Ins and D-chiro-Ins on oocyte quality and in vitro fertilization (IVF) outcomes [22, 23, 30].

Of note, two trials were randomized controlled Myo-Ins versus folic acid, as placebo [20, 25]; three were double-blind randomized controlled trial Myo-Ins versus folic acid [11, 21, 26]; one was a randomized controlled Myo-Ins versus metformin [27]. One study was a dose-response study of D-chiro-Ins on ovaries [28]. A single study, RCT, also compared the efficacy between Myo-Ins and D-chiro-Ins in improving oocyte quality [29]. In the last 3 RCTs, the combination of Myo-Ins/D-chiro-Ins (40 : 1) was examined in PCOS patients [22, 23, 30].

In the report of Genazzani et al. [20], PCOS patients were recruited in the trial and treated with either Myo-Ins plus folic acid (Inofolic®, LO.LI. Pharma, Rome, Italy) or folic acid alone (Table 1). The endocrine profile was evaluated and main outcomes are shown in Table 3. Consistent and significant changes were observed in the group receiving Myo-Ins plus folic acid. Indeed, prolactin (PRL), plasma luteinizing hormone (LH), and follicle stimulating hormone (FSH) ratio significantly decreased. The index of insulin sensitivity, expressed as glucose-to-insulin ratio, significantly increaed. The Ferriman-Gallwey score decreased after 12 weeks of Myo-Ins administration although the reduction was not statistically significant (22.7 ± 1.4 to 18.0 ± 0.8) whereas the reduction of the ovaries volumes was significant (12.2 ± 0.6 mL to 8.7 ± 0.8 mL, $p < 0.05$).

The study design and the endocrine profile after treatment obtained in the RCT of Costantino et al. [21] are shown in Tables 1 and 3. During the present study, a reduction in the systolic and diastolic blood pressure (SBP and DBP) values

Table 1: Eligible RCTs where myo-inositol and/or D-chiro-inositol have been evaluated for the treatment of PCOS patients.

Ref	Study design	Duration	Treatment	Number of subjects	Inclusion criteria	Exclusion criteria	Assessment of the response
[20]	Randomized, controlled	12 weeks	Treated group: Myo-Ins 2 g + FA 200 μg/d Control group: FA 200 μg/d	Number = 20 Treated: 10 Control: 10	PCOS, oligo/amenorrhea, normal PRL levels (range 5–25 ng/mL), and mild to severe hirsutism and/or acne	Hormone treatments in the last 24 weeks; adrenal enzymatic deficiency and/or other endocrine diseases	LH, FSH, PRL, E2, A, 17OHP, T, insulin, cortisol, OGTT[a] for insulin, glucose, C-peptide determinations, vaginal ultrasound examination Ferriman-Gallwey score, BMI, and HOMA index
[21]	Double-blind, randomized, controlled	12–16 weeks	Treated group: Myo-Ins 4 g + FA 400 μg/d Control group: FA 400 μg/d	Number = 42 Treated: 23 Control: 19	Age: <40 years PCOS, oligomenorrhea, and high serum-free T and/or hirsutism	Not described	Systolic/diastolic blood pressure, triglycerides, cholesterol, BMI, WHR, plasma glucose and insulin sensitivity, total/free T, DHEAS, SHBG, A, and progesterone peak value
[10]	Double-blind, randomized, controlled	16 weeks	Treated group: Myo-Ins 200 mg + FA 800 μg/d Control group: matching placebo	Number = 283 Treated: 136 Control: 147	Age: <35 years PCOS according to Adams et al. criteria[b], oligo/amenorrhea	Hyperprolactinemia, abnormal thyroid function tests, and congenital adrenal hyperplasia	E2, P and LH, BMI, ovulation frequency, inhibin-b, fasting glucose, fasting insulin, or insulin AUC, VLDL, LDL, HDL, total cholesterol, and triglycerides
[11]	Double-blind, randomized, controlled	16 weeks	Treated group: Myo-Ins 4 g + FA 400 μg/d Control group: FA 400 μg/d	Number = 92 Treated: 45 Control: 47	Age: <35 years PCOS according to Adams et al. criteria[b], oligo/amenorrhea	Hyperprolactinemia, abnormal thyroid function tests, and congenital adrenal hyperplasia	E2, P and LH, ratio of luteal phase weeks to observation weeks; inhibin-b, fasting glucose, fasting insulin, or insulin AUC, VLDL, LDL, HDL, total cholesterol, BMI, and triglycerides
[22]	Randomized controlled	24 weeks	Treated group: Myo-Ins 1.1 g + D-chiro-Ins 27.6 mg/d Control group: Myo-Ins 4 g/d	Number = 50 Treated: 26 Control: 24	Age: <41 years, BMI >27 kg/m^2, and PCOS according to Rotterdam criteria	Diabetic subjects, smokers, and alcohol users	Blood pressure, BMI, WHR, SHBG, serum steroids and lipid profile levels, OGTT, plasma glucose insulin, HOMA, and P
[23]	Randomized controlled	24 weeks	Treated group: Myo-Ins 1.1 g + D-chiro-Ins 27.6 mg + FA 400 μg/d Control group: FA 400 μg/d	Number = 46 Treated: 21 Control: 25	Age: <35 years, BMI >30 kg/m^2, and PCOS according to Rotterdam criteria	Diabetic subjects, smokers, and alcohol users	FSH, LH, E2, SHBG, A, free T, DHEA-S, HOMA index, and fasting glucose and insulin

Myo-Ins, myo-inositol; D-chiro-Ins, D-chiro-inositol; FA, folic acid; PCOS, polycystic ovary syndrome; PRL, prolactin; E2, oestradiol; A, androstenedione; 17OHP, 17-hydroxyprogesterone; T, testosterone; P, progesterone; OGTT, oral glucose tolerance; BMI, body mass index; LH, luteinizing hormone; FSH, follicle stimulating hormone; DHEAS, dehydroepiandrosterone; SHBG, sex hormone binding globulin; AUC, area under the curve of OGTT; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; WHR, waist-to-hip ratio.

[a]OGTT performed sampling 15 minutes before and 30, 60, 90, 120, and 240 minutes after the oral assumption of 75 g of glucose.

[b]Adams et al. [24].

Table 2: Eligible RCTs where myo-inositol and/or D-chiro-inositol have been evaluated for the treatment of PCOS patients undergoing ART.

Ref	Study design	Duration	Treatment	Number of subjects	Inclusion criteria	Exclusion criteria	Assessment of the response
[25]	Randomized, controlled	During ovulation induction for ICSI	Treated group: Myo-Ins 4 g + FA 400 μg/d Control group: FA 400 μg/d	Number = 60 Treated: 30 Control: 30	Age: <40 years, PCOS, oligo/amenorrhea, hyperandrogenism, hyperandrogenemia, typical features of ovaries on ultrasound scan	Hyperinsulinemia, hyperprolactinemia, hypothyroidism, androgen excess due to adrenal hyperplasia or Cushing syndrome	E2, stimulation (days), FSH IU Number of retrieved oocytes Number of MII, number of immature oocytes Number of embryos grade 1, embryo cleavage rate, fertilization rate Number of biochemical pregnancies Number of abortion cancellation rate Ovarian hyperstimulation syndrome
[26]	Double-blind randomized controlled	12 weeks	Treated group: Myo-Ins 4 g + FA 400 μg/d Control group: FA 400 μg/d	Number = 34 Treated: 17 Control: 17	Age: <40 years, PCOS, oligo/amenorrhea, hyperandrogenism, hyperandrogenemia, typical features of ovaries on ultrasound scan	Hypothyroidism, hyperthyroidism, diabetes mellitus, androgen-secreting cancers, adrenal hyperplasia, Cushing syndrome	E2, total r-FSH Number of follicles with a diameter >15 mm Number of oocytes retrieved Number of immature oocytes Number of embryos grade 1 Number of transferred embryos Number of biochemical pregnancies
[27]	Randomized, controlled	24 weeks	Treated group: Myo-Ins 4 g + FA 400 μg/d Control group: metformin 1.5 g/d	Number = 120 Treated: 60 Control: 60	Age: <35 years, PCOS according to Rotterdam criteria	Hyperprolactinemia, hypothyroidism, androgen excess, adrenal hyperplasia or Cushing's syndrome, tubal defects, semen parameters defects	Restoration of spontaneous ovarian activity by weekly serum P dosage and a transvaginal ultrasound scan documenting the presence of follicular growth or luteal cyst Number of pregnancies Abortion rate
[28]	Randomized controlled	8 weeks before r-FSH	Treated group: D-chiro-Ins 300, 600, 1200, and 2400 mg/d Control group: placebo	Number = 54 Treated: 4 groups (10–12 pts) Control: 11	Age: <40 years, PCOS according to Rotterdam criteria, undergoing ICSI procedure	Insulin resistance and/or hyperglycaemia	Total r-FSH, E2, stimulation (days) Number of oocytes retrieved Number of cycles cancelled Number of MII, number of immature oocytes Number of embryos grade 1
[29]	Randomized controlled	8 weeks before r-FSH	Treated group: Myo-Ins 4 g/d Control group: D-chiro-Ins 1.2 g/d	Number = 84 Treated: 43 Control: 41	Age: <40 years, PCOS according to Rotterdam criteria, undergoing ICSI procedure	Insulin resistance and/or hyperglycaemia	Duration of infertility, BMI, PRL, TSH, E2, stimulation (days), FSH Number of cancelled cycles Number of retrieved oocytes Number of MII, number of immature oocytes Number of embryos grade 1 Number of biochemical/clinical pregnancies Number of spontaneous abortions
[30]	Randomized controlled	12 weeks before r-FSH	Treated group: Myo-Ins 1.1 g + D-chiro-Ins 27.6 mg/d Control group: D-chiro-Ins 1 g/d	Number = 100 Treated: 47 Control: 53	Age: ≤35 years, >35 years BMI <28 kg/m^2, FSH <10 IU/L PCOS according to Rotterdam 2003 and a normal uterine cavity	Advanced stage (III or IV) endometriosis Poor responders pts or suffering from premature ovarian failure	Total IU of r-FSH, E2 before hCG injection Number of MII, number of VG-DEG Number of embryos grade 1 Number of embryos transferred Maturation rate and fertilization rate

Myo-Ins, myo-inositol; D-chiro-Ins, D-chiro-inositol; FA, folic acid; PCOS, polycystic ovary syndrome; E2, oestradiol; r-FSH, recombinant follicle stimulating hormone; MII, mature oocytes; VG-DEG, immature oocytes and degenerated oocytes; hCG, Human Chorionic Gonadotropin; ART, assisted reproductive technology.

Table 3: Biochemical and clinical findings related to hyperandrogenism and metabolism.

Ref	Treatment	Testosterone (ng/dL)	Androstenedione (ng/mL)	Free testosterone (ng/dL)	Insulin (μU/mL)	HOMA index	OGTT[a]	SHBG (nmol/L)	General findings
[20][c]	Myo-Ins versus FA	54.8 ± 6.2 versus 55.2 ± 9.1	1.70 ± 0.29 versus 1.91 ± 0.24	NA	6.5 ± 1.1***§§§ versus 11.3 ± 1.1	1.4 ± 0.3**§§ versus 2.5 ± 0.7	Myo-Ins improved glucose tolerance	NA	Myo-Ins significantly reduced LH, PRL, insulin levels, and LH/FSH ratio and significantly improved insulin sensitivity and menstrual cyclicity was restored in amenorrheic and oligomenorrheic subjects.
[21][c]	Myo-Ins versus FA	34.8 ± 4.3§§ versus 109.0 ± 7.5	1.96 ± 0.26 versus 3.06 ± 0.41	0.24 ± 0.03§§ versus 0.85 ± 0.13	26.0 ± 8.0 versus 38.0 ± 7.0	NA	Myo-Ins improved glucose tolerance	198.0 ± 24.0 versus 163.0 ± 26.0	Myo-Ins increased insulin sensitivity and improved glucose tolerance and insulin release. There was a significant reduction in total and free T. There was a decrement in systolic and diastolic blood pressure. Plasma triglycerides and total cholesterol concentration decreased.
[10][d]	Myo-Ins versus placebo	101.0 (81–121)§ versus 121.0 (101–141)	Decreased in Myo-Ins group	NA	No significant difference	NA	No significant difference	36.5§ versus 26.3	Myo-Ins showed a beneficial effect in improving ovarian function in PCOS women with oligomenorrhea.
[11][d]	Myo-Ins versus FA	95.0 (72–115) versus 118.0 (98–138)	Decreased in Myo-Ins group	NA	16.8 versus 17.3	NA	No significant difference	35.9§ versus 25.8	Myo-Ins treatment showed a beneficial effect in improving ovarian function, anthropometric measures, and lipid profile.
[22]	Myo-Ins + D-chiro-Ins versus Myo-Ins	32.7 ± 10.0** versus 40.1 ± 9.5**	1.94 ± 0.15** versus 1.98 ± 0.19**	0.23 ± 0.02** versus 0.24 ± 0.03**	9.2 ± 2.1** versus 9.6 ± 1.9**	1.5 ± 0.28** versus 1.9 ± 2.1**	Myo-Ins + D-chiro-Ins improved glucose tolerance	208 ± 20* versus 202 ± 27*	Both treatments, Myo-Ins + D-chiro-Ins, or Myo-Ins alone normalized the metabolic parameters and restored ovulation in overweight PCOS women. At the end of the treatment both the fasting insulin and glucose serum concentration level were significantly reduced. However, compared to Myo-Ins alone, the combined treatment has shown significant changes on the metabolic profile after only 12 weeks.

Table 3: Continued.

Ref	Treatment	Testosterone (ng/dL)	Androstenedione (ng/mL)	Free testosterone (ng/dL)	Insulin (μU/mL)	HOMA index	OGTT[a]	SHBG (nmol/L)	General findings
[23]	Myo-Ins + D-chiro-Ins versus Myo-Ins	NA	4.01 ± 1.7 versus 3.12 ± 2.23	0.62 ± 0.15* versus 0.83 ± 0.2	10.7 ± 5.5*** versus 17.8 ± 8.2	1.97 ± 1.48* versus 2.8 ± 1.4	NA	35.85 ± 24.3* versus 21.36 ± 7.57	Myo-Ins + D-chiro-Ins decreased significantly LH, free T levels, HOMA index, and fasting insulin. The combined treatment significantly increased E2 and SHBG. No relevant side effects were recorded. Therefore, the combined treatment, Myo-Ins + D-chiro-Ins, is effective in improving endocrine and metabolic parameters in young obese PCOS women.

Myo-Ins, myo-inositol; D-chiro-Ins, D-chiro-inositol; FA, folic acid; PCOS, polycystic ovary syndrome; PRL, prolactin; E2, oestradiol; A, androstenedione; 17OHP, 17-hydroxyprogesterone; T, testosterone; P, progesterone; OGTT, oral glucose tolerance; BMI, body mass index; LH, luteinizing hormone; FSH, follicle stimulating hormone; DHEAS, dehydroepiandrosterone; SHBG, sex hormone binding globulin; AUC, area under the curve of OGTT; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; WHR, waist-to-hip ratio.

[a]OGTT performed sampling 15 minutes before and 30, 60, 90, 120, and 240 minutes after the oral assumption of 75 g of glucose.

Values are mean ± SD. [c]Values are mean ± SEM. [d]Values are mean (CIs), confidence intervals (95%). A brief description is inserted in the table when numerical data are not available in the original article. The units were made uniform to show more comparable results.

p value: $\leq 0.05^{\S}$; $\leq 0.01^{\S\S}$; $\leq 0.001^{\S\S\S}$: comparison posttreatment experimental group versus control.

p value: $\leq 0.05^{*}$; $\leq 0.01^{**}$; $\leq 0.001^{***}$: comparison posttreatment with respect to baseline. Data at baseline are not shown in the table.

Table 4: IVF parameters and fertilization outcomes.

Ref	Treatment	E2 (pg/mL)	r-FSH dose (IU)	Stimulation days	MII	Oocyte retrieved	Embryo grade 1	Biochemical pregnancy (%)	Pregnancy rate (%)	General findings
[25]	Myo-Ins versus FA	2,232 ± 510[§] versus 2,713 ± 595	1,958 ± 695[§] versus 2,383 ± 578	11.4 ± 0.9[§] versus 12.4 ± 1.4	7.14 ± 3.49 versus 7.07 ± 3.04	8.76 ± 4.12 versus 9.37 ± 3.31	0.86 ± 0.83 versus 0.81 ± 0.83	9.1 versus 10	14.6 versus 12.9	Myo-Ins significantly reduced E2 at hCG administration, total r-FSH units, number of stimulation days, and number of VG-DEG, with a trend for increased percentage of oocytes in MII. Number of oocytes retrieved did not differ in the 2 groups.
[26][e]	Myo-Ins versus FA	Reduced in Myo-Ins group versus control	Reduced in Myo-Ins group versus control	NA	82.24% versus 66.87%	12[§] versus 8.50	68.1%[§§] versus 29%	No differences	NA	Myo-Ins has a positive effect on mature oocytes development and reduction of E2 and total r-FSH. Number of follicles with a diameter >15 mm visible at ultrasound scan during stimulation and the number of oocytes retrieved at the pick-up resulted significantly higher in the Myo-Ins-treated group. The number of immature oocytes was significantly reduced, and there was an increasing trend of the rate of oocytes in MII.
[27]	Myo-Ins versus metformin	NA	3 cycles × 37.5 U/day (if no pregnancy occurred)	NA	NA	NA	NA	30 versus 18.3	48.3 versus 36.6	Both Myo-Ins and metformin can be considered as first-line treatment for restoring normal menstrual cycles in most patients with PCOS; however Myo-Ins treatment seems to be more effective than metformin.
[28]	D-chiro-Ins (2400 mg) versus placebo	1,490.24 ± 253.21[§] versus 1,429.69 ± 1,118.43	2,983.0 ± 219.80[§§] versus 2,239.7 ± 181.55	13.8 ± 0.87[§§] versus 11.4 ± 1.2	Decreased progressively after D-chiro-Ins administration	No differences	Decreased progressively after D-chiro-Ins administration	NA	NA	High D-chiro-Ins dosage negatively affects oocyte quality. It worsens oocyte quality and ovarian response in nonobese and non-insulin resistant PCOS women.
[29]	Myo-Ins versus D-chiro-Ins	2,261.2 ± 456.6[§§] versus 2,740 ± 396.67	1,953.6 ± 397.5[§§] versus 2,360.5 ± 301.9	11.1 ± 0.8[§§] versus 12.7 ± 1.1	8.21 ± 2.39[§] versus 7.08 ± 2.67	8.90 ± 2.84 versus 9.32 ± 3.15	1.64 ± 0.88[§§] versus 0.76 ± 0.43	14 versus 9	51[§] versus 24	Myo-Ins significantly increased number of MII and decreased number of immature oocytes compared to D-chiro-Ins. Furthermore, it increased the mean number of top quality embryos and the total number of pregnancies compared to D-chiro-Ins. Number of oocytes retrieved did not differ in the two treatments groups.
[30]	Myo-Ins + D-chiro-Ins versus D-chiro-Ins	Age ≤35	2,230.09 ± 827.57 versus 2,537.94 ± 860.19	1,569.02 ± 497.12[§] versus 1,899.21 ± 618.17	NA	7.91 ± 4.51 versus 8.00 ± 3.92	9.91 ± 4.85 versus 10.79 ± 4.66	0.96 ± 0.83[§§§] versus 0.73 ± 0.73	NA	NA	The combined treatment with Myo-Ins + D-chiro-Ins, rather than D-chiro-Ins alone, was able to improve oocyte quality and high-quality embryos in PCOS women undergoing ART regardless of the age.
		Age ≥35	2,185.09 ± 409.08[§] versus 2,519.85 ± 788.49	1,906.96 ± 770.59 versus 2,170.58 ± 694.44	NA	6.91 ± 2.26 versus 8.35 ± 5.19	8.35 ± 3.21[§] versus 10.75 ± 5.23	0.90 ± 0.80[§] versus 0.68 ± 0.80	NA	NA	

Myo-Ins, myo-inositol; D-chiro-Ins, D-chiro-inositol; FA, folic acid; PCOS, polycystic ovary syndrome; E2, oestradiol; r-FSH, recombinant follicle stimulating hormone; MII, mature oocytes; VG-DEG, immature oocytes and degenerated oocytes; hCG, Human Chorionic Gonadotropin; ART, assisted reproductive technology.
Values are mean ± SD. [e]Values are shown as median. A brief description is inserted in the table when numerical data are not available.
p value: ≤0.05[§]; ≤0.01[§§]; ≤0.001[§§§]: comparison posttreatment experimental group versus control.

was observed in patients treated with Myo-Ins (131±2 mmHg to 127 ± 2 mmHg and 88 ± 1 mmHg to 82 ± 3 mmHg, resp.), while these values increased in placebo group (128 ± 1 mmHg to 130 ± 1 mmHg, $p = 0.002$, and 86 ± 7 mmHg to 90 ± 1 mmHg, $p = 0.001$, resp.). Furthermore, in Myo-Ins group, plasma triglycerides decreased from 195 ± 20 mg/dL to 95 ± 17 mg/dL and total cholesterol significantly decreased from 210±10 mg/dL to 171±11 mg/dL. In Myo-Ins-treated group the composite whole body insulin sensitivity index (ISI) increased significantly from 2.80 ± 0.35 mg/dL to 5.05 ± 0.59 mg/dL, while it did not change in placebo group. Ovulation was restored in 69.5% of women in Myo-Ins group and 21% of placebo ($p = 0.001$). After treatment, the peak level of progesterone (P) was higher in Myo-Ins patients (15.1 ± 2.2 ng/mL) compared to placebo. Furthermore, there was a significant reduction of more than 50% in the serum dehydroepiandrosterone sulphate in Myo-Ins women (366 ± 47 μg/dL to 188 ± 24 μg/dL; $p = 0.003$), whereas it was not significant in placebo.

Gerli et al. [10, 11] evaluated the effect of Myo-Ins on ovarian and metabolic factors in PCOS subjects, in 2 different studies conducted in 2003 and 2007 (Tables 1 and 3); in the first trial [10], the ovulation frequency was significantly higher ($p < 0.01$) in Myo-Ins-treated group (23%) compared with placebo (13%). The main outcomes are defined in Table 3. In addition, it was found that E2 concentration increased only in Myo-Ins group during the first week of treatment inducing follicular maturation. The body mass index (BMI) and leptin were significantly reduced in treated patients, whereas body weight augmented in placebo. A significant increase in circulating high-density lipoprotein (HDL) was recorded in Myo-Ins women. In the second study [11], in addition to the main findings shown in Table 3, a significant increment of the ovulation frequency in Myo-Ins group compared to placebo was observed. All patients started treatment outside the luteal phase, and the delay to the first ovulation after starting the program was significantly shorter in the study group (24.5 versus 40.5, $p = 0.02$). The analysis on the first and eighth day of treatment showed that the Myo-Ins-treated group had a significant increase in E2 levels ($p = 0.03$), whereas controls showed no change. Circulating levels of inhibin B remained unvaried. Circulating leptin concentration declined in Myo-Ins patients, in contrast to controls. The low-density lipoprotein (LDL) showed a trend toward reduction, and the HDL increased significantly in Myo-Ins group.

In Nordio and Proietti study [22], the combination of Myo-Ins and D-chiro-Ins versus Myo-Ins alone was evaluated (Tables 1 and 3). Either treatment was efficacious in improving the ovulation function and metabolic parameters. Besides the main findings displayed in Table 3, a reduction of SBP and SDB was observed in both groups (Myo-Ins plus D-chiro-Ins, 131.0 ± 1.6 mmHg to 128.0 ± 1.2 mmHg and 88.0 ± 3.3 to 80 ± 2 mmHg, resp., versus Myo-Ins, 129.0 ± 2.5 mmHg to 127 ± 2 mmHg and 87.0 ± 2.6 mmHg to 82 ± 1 mmHg, resp.). Also BMI and waist-to-hip ratio (WHR) were reduced after treatment but not significantly.

In a very recent study [23], an improvement of patients' insulin resistance and ovulatory function was observed after Myo-Ins and D-chiro-Ins treatment, significantly rebalancing their endocrine and metabolic profiles (Tables 1 and 3).

Papaleo et al. [25] broaden the clinical use of Myo-Ins by evaluating its effect on oocyte quality and the ovarian stimulation protocol for PCOS women (Table 2). As can be seen in Table 4, the number of oocytes retrieved did not differ between the two groups, whereas in the group treated with Myo-Ins the number of immature oocytes and degenerated oocytes was significantly reduced (1.0 ± 0.9 versus 1.6 ± 1.0; $p = 0.01$), with a trend for increased percentage of metaphase II stage oocytes.

In the study of Ciotta et al. [26], oocyte's quality was assessed after the oocyte pick-up during the assisted reproductive technology (ART) procedure in women with PCOS (Table 2). Besides results shown in Table 4, the number of immature oocytes resulting significantly reduced in Myo-Ins group (degenerated oocytes 0.93% versus 14.37%, $p < 0.02$; germinal vesicles 1.4% versus 9.37%, $p < 0.02$) and the mean number of transferred embryos was significantly higher.

Raffone et al. [27] compared the effects of metformin (Glucophage®, Merck Pharma) and Myo-Ins (Inofolic, LO.LI. Pharma, Rome, Italy) on PCOS patients (Tables 2 and 4). In Myo-Ins group 65% of patients versus 50% of metformin group restored spontaneous ovulation activity, after a mean of 14.8 (±1.8) days and 16.7 (±2.5) days from day 1 of the menstrual cycle, respectively.

Fifty-four women diagnosed with PCOS were selected in the study of Isabella and Raffone, 2012 [28] (Table 2). Patients were randomized into 5 groups, including a placebo group and 4 groups that received 300, 600, 1200, and 2400 mg of D-chiro-Ins (Interquim s.a., Barcelona, Spain) daily, respectively. In addition to the main results reported in Table 4, they found that high D-chiro-Ins concentrations progressively increase the number of immature oocytes, in a significant manner ($p < 0.04$).

As shown in Tables 2 and 4, Unfer et al. [29] compared the efficacy of Myo-Ins and D-chiro-Ins in patients diagnosed with PCOS. The selected ones were randomly divided into two groups receiving either Myo-Ins or D-chiro-Ins (Table 2). Along with the main findings presented in Table 4, it was reported that the number of immature oocytes was significantly lower in Myo-Ins group compared to D-chiro-Ins group (0.69 ± 0.64 versus 2.23 ± 0.85; $p < 0.01$).

The combination 40 : 1 of Myo-Ins and D-chiro-Ins (Inofolic Combi, LO.LI. Pharma, Rome, Italy; patented) was also evaluated by Colazingari et al. [30], in PCOS patients undergoing IVF (Table 2). In this study, for evaluation of results, women age was also taken into account, dividing them into 2 further categories: ≤35 or >35 years. The combination of Myo-Ins and D-chiro-Ins gave a greater result in the ovarian stimulation protocol compared to D-chiro-Ins alone (Table 4). In Myo-Ins plus D-chiro-Ins patients, oocytes of high quality resulted and the number of degenerated oocytes was lower. In particular results showed that Myo-Ins plus D-chiro-Ins treatment reduced the number of degenerated oocytes in both age groups (≤35 years old: 1.04 ± 1.15 versus 1.82 ± 1.55; >35 years old: 1.00 ± 0.91 versus 1.45 ± 0.89).

4. Discussion

A critical review of the 12 RCTs included in this systematic review highlights that oral administration of Myo-Ins, alone or in combination with D-chiro-Ins, is capable of restoring spontaneous ovulation and improving fertility in women with PCOS.

Myo-Ins and D-chiro-Ins are 2 of the 9 different stereoisomers of inositol, polyol found in many foods, in particular cereals, nuts, and fruits as well as in human cells. They exert important actions in the control of glucose homeostasis and, when incorporated into phosphoglycans, have been shown to serve as second messengers involved in the signaling-transduction cascade of insulin [36, 37]; Myo-Ins and D-chiro-Ins are also involved in a number of biochemical pathways within oocytes [38, 39]. PCOS women have lower serum D-chiro-Ins levels and elevated urinary loss of D-chiro-Ins-IPG [40]. As noted above, inositol phosphoglycans (IPGs) are potentially important putative intracellular mediators of insulin action. It has been demonstrated that, in patients affected by PCOS, the metabolism of inositol is dysregulated, highlighting the subtle connection between insulin resistance and inositol deficiency in PCOS patients [41]. Indeed, in women with PCOS, insulin resistance and compensatory hyperinsulinemia due to dysregulation of inositol metabolism may actually be the major underlying cause of the disorder. Various studies have shown the role of D-chiro-Ins at low dosage in increasing insulin sensitivity and ovulation frequency, as well as in decreasing levels of lipid biomarkers and serum androgen [12, 13]. D-chiro-Ins is converted from Myo-Ins through insulin-stimulated NAD-dependent epimerase. Myo-Ins is the most abundant inositol isomer within the ovary, as suggested by the fact that approximately 99% of the ovarian intracellular pool of inositol consists of Myo-Ins [17]. Indeed, it was shown that an increased activity of epimerase in theca cells of ovaries of PCOS women is associated with a consistent reduction in the intraovarian ratio of Myo-Ins to D-chiro-Ins [19]. These experimental data are in line with the so-called D-chiro-Ins ovarian paradox posited by Carlomagno et al. [18]; these investigators advanced the hypothesis that epimerase activity is increased in the ovaries of PCOS subjects, resulting in a local Myo-Ins deficiency responsible for the oligoovulation and poor oocyte quality of the disorder. This hypothesis has drawn attention to the importance of Myo-Ins and D-chiro-Ins supplementation in a physiological ratio in order to restore normal ovary functionality. In fact, a correlation between Myo-Ins concentration in the follicular fluid and high oocyte quality was found and a number of studies have reported that Myo-Ins supplementation is able to improve oocyte quality [25, 31].

In this systematic review a number of recent articles were selected in order to critically analyze the roles of Myo-Ins and D-chiro-Ins, combined or alone, as a treatment of PCOS. Although there are a number of published articles on the use of Myo-Ins as a treatment in women with PCOS, only few of them were designed as RCT. These RCT studies, reviewed here, support the hypothesis of a primary role of IPGs as second messengers of insulin signaling and demonstrate that Myo-Ins supplementation beneficially affects the hormonal milieu of PCOS patients. Indeed, these trials provide evidence that Myo-Ins reduces insulin levels, probably either by conversion to D-chiro-Ins (via the epimerase enzyme) or by serving as substrate for the formation of Myo-Ins-containing IPGs and D-chiro-Ins-containing IPGs, which would in turn amplify insulin signaling. In particular, two studies [20, 25] suggest that deficiency of Myo-Ins and/or D-chiro-Ins might be an additional cofactor contributing to the pathophysiology of the insulin resistance of PCOS patients [42]. In these studies, hormonal parameters improved significantly in all PCOS patients treated with Myo-Ins [10, 11, 20, 21, 25–27, 29]. In a study by Gerli et al. body weight and circulating leptin decreased significantly and HDL concentrations increased significantly in the patients treated with Myo-Ins, compared with the placebo group, providing the first indication that Myo-Ins treatment might possibly reduce the risk of cardiovascular diseases in PCOS women. Moreover, in an equivalency study, Raffone et al. [27] stated that Myo-Ins improves the pregnancy rate in PCOS women. These findings further support the hypothesis of a key role of IPG as second messenger of insulin signaling. The oral supplementation of Myo-Ins might reduce insulin levels, by providing a higher availability of IPG precursors, in this way improving the activities of this second messenger of insulin signal [27].

The study by Ciotta et al. demonstrated that Myo-Ins treatment reduced the number of germinal vesicles and degenerated oocytes and improved the development of mature oocytes, as previously reported in experimental data [43]. The authors concluded that Myo-Ins alone is useful in PCOS patients as insulin-sensitizer and for induction of oocyte maturation [26], confirming that Myo-Ins is likely an important constituent of the follicular microenvironment for normal nuclear and cytoplasmic oocyte's development.

As already noted, the role played by D-chiro-Ins in ovarian physiology is controversial. In this regard, a study in which different concentrations of D-chiro-Ins were administrated to nonobese PCOS women with normal insulin sensitivity undergoing IVF reported that as the dosage of D-chiro-Ins was progressively increased, oocyte quality and ovarian response worsened [28]. A possible explanation for this observation may lie in the different tissue-specific ratios of Myo-Ins/D-chiro-Ins in different organs (i.e., 100 : 1 in the ovary) and the diverse physiological roles of inositol stereoisomers, as Myo-Ins increases glucose cellular uptake and D-chiro-Ins is involved in glycogen synthesis [33, 44]. In fact, cells responsible for glycogen storage (such as liver, muscles, and fat cells) contain high levels of D-chiro-Ins, whereas brain and heart cells contain high concentration of Myo-Ins, since they require high consumption of glucose. These data are in line with the D-chiro-Ins paradox hypothesis and with the data of Unfer et al. (2011) that demonstrated that Myo-Ins rather than D-chiro-Ins improved oocyte quality in intracytoplasmic sperm injection cycles [18, 29]. To wit, Unfer et al. demonstrated that Myo-Ins treatment significantly reduced ovarian stimulation days and the IU of r-FSH administrated and improved both oocyte and embryo quality in euglycemic PCOS patients when compared with treatment with D-chiro-Ins. This was also shown in 2009 by Papaleo

et al. and included in our previous systematic review [14, 25]. However, as demonstrated by Nordio and Proietti, the combination of Myo-Ins and D-chiro-Ins, at a physiological ratio of 40 : 1, was able to more quickly restore to normal the hormonal and metabolic parameters in overweight PCOS women than Myo-Ins treatment alone [22]. Bearing in mind previous studies, the physiological ratio of these two isomers (40 : 1) seems to be an optimal and promising approach for the treatment of PCOS disorders [45, 46].

This might be due to the synergistic action of Myo-Ins and D-chiro-Ins, as they regulate different biological processes. In fact, the combination of Myo-Ins and D-chiro-Ins may be particularly beneficial in overweight PCOS women, considering that Myo-Ins improves the ovulatory function and D-chiro-Ins rapidly reduces the peripheral hyperinsulinemia. Notably, Colazingari et al. also reported that combined therapy of Myo-Ins and D-chiro-Ins, rather than D-chiro-Ins alone, improved oocyte quality in PCOS women undergoing ART [30]. This study further corroborates previous data, suggesting that D-chiro-Ins supplementation alone might not be the optimal or appropriate approach for improving IVF outcomes in PCOS patients.

Treatment with the combination of Myo-Ins and D-chiro-Ins has been further investigated by Benelli et al. who demonstrated that these two molecules, together in a 40 : 1 ratio, improved the endocrine profile and insulin resistance of obese women with PCOS [23]. An important aspect of this study was that no relevant side effects were recorded during combined therapy with Myo-Ins and D-chiro-Ins, providing further evidence of the safety of the usage of these two stereoisomers in combination. There is also accumulating evidence on the beneficial effects of Myo-Ins administration on reproductive function and the efficacy of combined Myo-Ins/D-chiro-Ins administration, in the physiological plasma ratio of 40 : 1, for amelioration of the metabolic aberrations of PCOS and for restoring spontaneous ovulation [47].

In conclusion, the analysis of these clinical trials highlights the salutary effects of Myo-Ins supplementation in improving several of the hormonal and reproductive disturbances of PCOS; furthermore, the analysis lends prominence to the pivotal role of inositol(s), mainly Myo-Ins and D-chiro-Ins, as a safe and effective therapy for PCOS, including an enhanced oocyte follicular development and oocyte maturation and in stimulation and pregnancy outcomes in IVF procedures.

Competing Interests

Vittorio Unfer is employee at LO.LI. Pharma, Rome, Italy. The other authors declare that they have no conflict of interests regarding the publication of this paper.

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Research Article

The Effectiveness of Myo-Inositol and D-Chiro Inositol Treatment in Type 2 Diabetes

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Inositol has been used as a supplement in treating several pathologies such as PCOS, metabolic syndrome, and gestational diabetes. Both myo-inositol and its isomer d-chiro-inositol showed insulin mimetic effects in conditions of insulin resistance. Type 2 diabetes (T2DM) is a condition typically caused by insulin resistance. There is a lack of evidence of inositol use in T2DM. We evaluated the effectiveness and safety of myo-inositol and d-chiro-inositol treatment in T2DM. This was a pilot study involving a consecutive sample of patients with T2DM with suboptimal glycemic control (HbA1c 7.0–10.0%) already treated with glucose-lowering agents. Patients (23.1% males, mean age of 60.8 ± 11.7 years) took for three months a combination of myo-inositol (550 mg) and d-chiro-inositol (13.8 mg) orally twice a day as add-on supplement to their glucose-lowering drugs. Possible occurrence of side effects was investigated. After three months of treatment fasting blood glucose (192.6 ± 60.2 versus 160.9 ± 36.4; $p = 0.02$) and HbA1c levels (8.6 ± 0.9 versus 7.7 ± 0.9; $p = 0.02$) significantly decreased compared to baseline. There was no significant difference in blood pressure, lipid profile, and BMI levels. None of the participants reported side effects. In conclusion, a supplementation with a combination of myo- and d-chiro-inositol is an effective and safe strategy for improving glycemic control in T2DM.

1. Introduction

Inositol is a cyclitol present in animal and plant cells. It can be present in nine distinct stereoisomers, myo-inositol being the most represented. D-chiro-inositol is an inositol isoform derived from myo-inositol through an epimerization process and this reaction is insulin dependent [1]. Both myo- and d-chiro-inositol showed insulin mimetic effects in animal models of insulin resistance [2, 3]. They have been studied in basic research and their potential has been recognized as very promising [4]. A specific physiological myo-/d-chiro-inositol ratio exists in different human tissues [5]. The distinctive ratio depends on the specific biological function these molecules have. Indeed, looking at the molecular pathway of inositols, after insulin links with the cell, inositol-second messengers are produced. While d-chiro-inositol-based second messengers promote glycogen synthesis, second messengers based on myo-inositol regulate glucose intake increasing the activity of glucose transport proteins. Recently, a new supplement formulation conforming to this physiological ratio has been designed [6, 7]. Given inositol pivotal role in regulating many metabolic pathways and hormonal signalling, its use in clinical settings is steeply growing. Inositol has been mainly used as a supplement in treating several pathologies such as PCOS [8], metabolic syndrome [9, 10], and gestational diabetes (GDM) [11]. In the case of GDM, a condition defined as a glucose impairment first detected in pregnancy, a preventive role of inositol for GDM onset was recognized [12–14]. In addition, inositol has been studied as a therapeutic option for the treatment of GDM [15, 16]. The main effect of inositol when used in pregnancy is decreasing the level of insulin resistance. Consequently, a potential role of inositol as a treatment option could be hypothesized for other conditions typically characterized by insulin resistance. The most common disease in which insulin resistance represents a significant factor is type 2 diabetes mellitus (T2DM). It is characterized by a double pathophysiological alteration represented on one side by an increase in insulin resistance, that is, the inability of the action of blood circulating insulin, due to a resistance of the target tissues; on the other side, by

a deficiency of insulin secretion from the pancreas [17]. The fist-line therapy for treating T2DM is metformin, an insulin sensitizer drug [18, 19]. However, the natural history of the disease implies that the therapy with only one oral hypoglycemic agent may be no longer able to guarantee adequate glycemic compensation. It therefore becomes necessary to start a therapy with a combination of other glucose-lowering drugs and, in the case of failure of oral therapy, to start insulin [20, 21]. There is a lack of evidence of inositol use in T2DM. Inositol could represent a valid strategy in treating T2DM in addiction to hypoglycemic drugs. Aim of our study was to evaluate the effectiveness and safety of myo-inositol and d-chiro-inositol treatment in T2DM.

2. Materials and Methods

This was a pilot study involving people with T2DM cared for at the diabetes outpatient unit of the Niguarda Cà Granda Hospital, Milan, Italy. A consecutive sample of patients with T2DM treated with at least one glucose-lowering agent was included in the study, irrespective of gender and diabetes duration. Patients were not referred to the center at the time they entered the study; they instead had received a medical continuous assistance, being treated at the same center for at least one year. Subjects were included in the study if their glucose status was steadily suboptimal for at least 3 months. Suboptimal glycemic control was defined as a glycated hemoglobin (HbA1c) between 7.0% (53 mmol/mol) and 10.0% (86 mmol/mol). Other inclusion criteria were age ≥ 18 years, unchanged glucose-lowering treatment in the last 3 months, and informed consent signature. Exclusion criteria were the presence of renal or liver diseases, severe heart failure, psychiatric disease, and pregnancy.

To all the involved subjects a combination of myo-inositol (550 mg), d-chiro-inositol (13.8 mg), and folic acid (400 mcg) (INOFOLIC® Combi, soft gel, Lo.Li. Pharma, Rome, Italy) was suggested to be taken orally twice a day as add-on supplement to their glucose-lowering drugs. All participants, as standard procedure at each visit, were properly advised to follow a hypocaloric and low-glycemic index diet according to standard care and to maintain their standard physical activity. For all patients a three-month follow-up visit was scheduled. Clinical information at baseline and after three months visits was recorded. Specifically, information on the following parameters was collected: age, gender, diabetes duration, height, weight, smoke habits, blood pressure levels, HbA1c levels, lipid profile, pharmacologic treatments (antihypertensive, lipid-lowering, and glucose-lowering drugs), and diabetes-related complications such as retinopathy, nephropathy, neuropathy, and macroangiopathy. In particular, macroangiopathy was defined as a history of a cardiovascular event and/or ischemic electrocardiogram abnormalities at rest or in a stress test, or the presence of plaques detected by ultrasonographic examination of the carotid arteries or the peripheral arterial vessels or as the presence of an intima media of thickness >1.5 mm [22]; neuropathy was diagnosed by the vibration perception test, the monofilament pressure sensation test, or electromyography; nephropathy was defined as an increased urinary albumin excretion (albuminuria) diagnosed if urinary albumin concentration was >30 mg/l, or if urinary albumin excretion rate was >20 μg/min, or if urinary albumin-to-creatinine ratio was >2.5 mg/mmol in men and 3.5 mg/mmol in women; and retinopathy was detected by high-quality fundus photographs [23].

Possible occurrence of side effects related to the consumption of inositol was investigated at each visit. All the clinical data collected in the study have been analyzed anonymously. Local Ethic Committee approved the protocol and all participating subjects gave a written informed consent. The study was conducted according to the Helsinki Declaration.

2.1. Statistical Analyses. Data were expressed as means ± standard deviation for continuous variables and percentages for categorical variables. The Kolmogorov–Smirnov test was used to test the normality of distribution of continuous variables. Clinical and demographic characteristics were compared using the Wilcoxon signed-rank test. As this was a feasibility study, no prior power calculation was performed. A p value < 0.05 was considered for statistical significance. Analyses were performed using SPSS version 21.0 (SPSS, Inc., Chicago, IL).

3. Results and Discussion

Overall, 20 subjects with T2DM were involved in the study. The acceptance rate of participation at the study of patients to which it was proposed was 100%. Demographic and clinical characteristics of the studied patients are reported in Table 1. One-fourth of participants were male. Almost half of the entire population had diabetes-related complications. Comorbidities such as antihypertensive and lipid disorders were present in almost half of the cases. With respect to diabetes treatment 53.8% of subjects were treated with only oral hypoglycemic agents (OHA), 38.5% with OHA plus insulin, and 7.7% with only insulin. After three months of treatment fasting blood glucose levels (192.6 ± 60.2 versus 160.9 ± 36.4, $p = 0.02$) and HbA1c levels (8.6 ± 0.9 versus 7.7 ± 0.9, $p = 0.02$) significantly decreased compared to baseline. HbA1c reduction was of −1.0 ± 1.5%. There was no statistically significant difference in systolic ($p = 0.10$) and diastolic ($p = 0.90$) blood pressure, lipid profile ($p = 0.31$ for total cholesterol; $p = 0.89$ for HDL cholesterol; $p = 0.61$ for triglycerides; $p = 0.69$ for LDL cholesterol), and BMI levels ($p = 0.14$) changes (Table 2). None of the participants reported side effects linked to the consumption of inositol.

The main finding of our study is that inositol could represent a valid strategy for improving glycemic control in T2DM, its supplementation being effective in lowering both fasting blood glucose and HbA1c levels. We cannot compare our results with existing literature because of the lack of studies involving people with T2DM treated with inositol. There is only evidence that both myo-inositol to chiro-inositol epimerase activities and chiro-inositol to myo-inositol ratios are decreased in tissues of GK type 2 diabetic rats, potentially playing a role in explaining the decreased chiro-inositol to

Table 1: Baseline demographic and clinical characteristics of the studied patients.

Gender (%)	
Male	23.1
Female	76.9
Age (years)	60.8 ± 11.7
Diabetes duration (years)	11.5 ± 7.6
Smokers (%)	23.1
Diabetes complications (%)	
None	53.8
Retinopathy	15.4
Nephropathy	15.4
Macroangiopathy	15.4
Neuropathy	0.0
Feet	0.0
Antihypertensive treatment (%)	53.8
Lipid-lowering treatment (%)	53.8

Table 2: Clinical changes between baseline and three months after starting inositol treatment.

	Baseline	3 months	p
Weight (kg)	80.5 ± 17.7	79.8 ± 15.8	0.14
BMI (Kg/m^2)	31.1 ± 5.9	30.9 ± 6.6	0.14
Classes of BMI (%):			0.34
<25 Kg/m^2	15.4	20.0	
25–29 Kg/m^2	23.1	10.0	
≥30 Kg/m^2	61.5	70.0	
Systolic blood pressure (mmHg)	126.7 ± 10.8	124.4 ± 9.5	0.10
Diastolic blood pressure (mmHg)	75.0 ± 7.7	76.1 ± 5.5	0.90
Fasting blood glucose levels (mg/dl)	192.6 ± 60.2	160.9 ± 36.4	0.02
HbA1c (%)	8.6 ± 0.9	7.7 ± 0.9	0.02
Total cholesterol (mg/dl)	189.3 ± 39.5	198.7 ± 38.8	0.31
HDL cholesterol (mg/dl)	57.3 ± 19.6	50.6 ± 10.1	0.89
Triglycerides (mg/dl)	161.5 ± 131.1	149.9 ± 64.4	0.61
LDL cholesterol (mg/dl)	98.9 ± 36.1	116.4 ± 43.7	0.69

myo-inositol urine and tissue ratios observed in animal and human studies [24]. This mechanism may also possibly play a role in explaining insulin resistance status. A tissue-specific myo-inositol/d-chiro-inositol ratio exists. This characteristic is strictly linked to the different biological actions that inositol isoforms exert, myo-inositol being more effective in increasing the insulin sensitivity level of typically insulin-dependent tissues (e.g., muscle, fat) and d-chiro-inositol more involved in tissues where glycogen synthesis takes place (e.g., liver). We did not use myo-inositol or d-chiro-inositol alone because we decided to administer inositol complying with a proportion that should reflect the natural balance among the two stereoisomers. This new formulation reflects the myo-inositol/d-chiro-inositol ratio of 40 : 1 that is the physiological ratio we can find in the plasma.

Our study was conducted involving people with T2DM, one of the largest global health emergencies with a huge economic impact. Its prevalence is increasing worldwide and it is expected that it will increase up to 7.7% in 2030. Each year more and more people live with this condition, which can result in life-changing complications. In addition to the 415 million adults who are estimated to currently have diabetes, there are 318 million adults with impaired glucose tolerance, which puts them at high risk of developing the disease in the future [25]. People with diabetes are at high risk of developing a number of disabling and life-threatening health problems. Diabetes complications can be prevented or delayed by maintaining blood glucose, blood pressure, and cholesterol levels as close to normal as possible. Several studies have shown a clear association between strict glucose control and a low risk of diabetes-related complications [26, 27]. The greatest clinical objective on both the part of health care professionals and patients is maintaining HbA1c in target, thus limiting the occurrence of complications.

The result of HbA1c reduction in our study was quite surprising because of the short period of treatment and because of the extent of the reduction. OHA can improve HbA1c levels with a mean HBA1c reduction of 1% [28, 29]. We found that a very important inositol effect on glycemic control exists. The reason for this could be likely linked to a lowering of the insulin resistance status thanks to the metabolic effect of inositol. However, we cannot exclude the possibility that a part of this effect could be associated only with lifestyle changes such as more proper diet and physical activity. We can exclude a putative effect on blood glucose control of folic acid contained in the supplement, as a systematic review and meta-analysis confirm [30].

Although our study was a pilot study, despite the small sample size, it reached a statistical significance in the two main parameters (i.e., blood glucose and HbA1c) defining glycemic control. Another novelty of our study was having tested the metabolic effect of inositol supplementation in a population including also males. The studies until now published on inositol have involved only women with several clinical (i.e., PCOS, metabolic syndrome, and GDM) or physiological (i.e., menopause) conditions. Only a single randomized, double-blind, placebo-controlled study involved a sample of males with T2MD but it was focused on investigating myoinositol use in the treatment of erectile dysfunction [31]. However, authors did not specifically investigate parameters linked to glycemic control. Our findings could extend the target population for inositol use also to males, its supplementation being effective in improving metabolic parameters. As an effective insulin sensitizer inositol could represent a possible alternative to metformin or pioglitazone that are typically used as insulin sensitizer glucose-lowering drugs, when their use is not possible (i.e., metformin intolerance, drugs contraindications).

Having carried out the study in the context of an usual care setting, we were not able to have information on insulin-resistance levels, serum insulin being a parameter not usually requested and used. This represents a limitation of our study.

4. Conclusions

Our study is important for several reasons. It showed for the first time a direct beneficial effect of the supplementation with the association of myo- and d-chiro-inositol on glycemic parameters of subjects with T2DM. Particularly, a significant reduction in blood glucose and HbA1c levels was registered. Inositol supplementation did not lead to any side effect, confirming the safety of this molecule. Our study findings are relevant, mostly for their possible clinical implication. Our data are promising but they need to be confirmed by studies with a larger sample size and a randomized controlled trial design.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Broad Spectrum Anticancer Activity of Myo-Inositol and Inositol Hexakisphosphate

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Inositols (myo-inositol and inositol hexakisphosphate) exert a wide range of critical activities in both physiological and pathological settings. Deregulated inositol metabolism has been recorded in a number of diseases, including cancer, where inositol modulates different critical pathways. Inositols inhibit pRB phosphorylation, fostering the pRB/E2F complexes formation and blocking progression along the cell cycle. Inositols reduce PI3K levels, thus counteracting the activation of the PKC/RAS/ERK pathway downstream of PI3K activation. Upstream of that pathway, inositols disrupt the ligand interaction between FGF and its receptor as well as with the EGF-transduction processes involving IGF-II receptor and AP-1 complexes. Additionally, Akt activation is severely impaired upon inositol addition. Downregulation of both Akt and ERK leads consequently to NF-kB inhibition and reduced expression of inflammatory markers (COX-2 and PGE2). Remarkably, inositol-induced downregulation of presenilin-1 interferes with the epithelial-mesenchymal transition and reduces Wnt-activation, β-catenin translocation, Notch-1, N-cadherin, and SNAI1 release. Inositols interfere also with the cytoskeleton by upregulating Focal Adhesion Kinase and E-cadherin and decreasing Fascin and Cofilin, two main components of pseudopodia, leading hence to invasiveness impairment. This effect is reinforced by the inositol-induced inhibition on metalloproteinases and ROCK1/2 release. Overall, these effects enable inositols to remodel the cytoskeleton architecture.

1. Introduction

Inositol (myo-Ins) and its phosphate metabolites exert a wide range of critical activities in both physiological and pathological settings. Indeed, deregulation of inositol metabolism has been extensively investigated in several illnesses, including neurological disorders [1], polycystic ovary syndrome [2], and metabolic diseases [3].

In the wake of the renewed interest for inositol phosphates (InsPs) and other inositol-based compounds, studies on the anticancer properties of both inositol hexakisphosphate (InsP6) and myo-Ins have gained momentum during the last decades. InsP6 inhibits growth and invasiveness of a number of cancer types, while both InsP6 and myo-Ins have been demonstrated to display significant chemopreventive effects both *in vitro* and *in vivo*. Furthermore, inositols have been involved in modulating unexpected processes, including mRNA transcription, chromatin remodeling, cytoskeleton configuration, and p53 activity, just to mention a few. Therefore, these new findings prompted reassessing under a new light the putative role of both myo-Ins and InsP6 in carcinogenesis.

2. Epidemiology: Diet and Cancer

Since the early eighties [4], it has been recognized that wide variation in cancer incidence among different countries around the world can primarily be ascribed to environmental

factors, among which diet is likely the most important [5]. This evidence is very strong for some cancers, like breast, prostate, and colon tumors, where differences in tumor incidence across countries have been mainly ascribed to their respective dietary habits [6]. Those data provided the rationale for the so-called "fiber hypotheses" [7], for which grains refining and lack of dietary fiber may have a "causative" role in colon and breast carcinogenesis [8]. Several investigations led support to this hypothesis [9], even if some inconsistencies have been recorded [10]. Yet, such association is probably a too simplistic one, given that fibers could not be the sole putative preventive factor. Indeed, colon cancer incidence has been shown to differ significantly among groups consuming approximately the same amount of fibers [11]. These findings indicate that to assess the correlation between diet and cancer properly we should evaluate the consumption of specific components rather than focusing on the overall fiber intake. Both epidemiological and molecular investigations have indeed provided valuable data suggesting that distinct dietary components may exert specific anticancer activities. Among those nutrients, compelling evidence gathered to date has evidenced that lignans, polyphenolic acids, stilbenes, bioflavonoids, phytic acid, and inositols exert unquestionable anticancer effects [12, 13]. Moreover, it has been observed that only the consumption of fibers with high content of phytic acid is inversely correlated with colon cancer [14].

3. Inositol and Inositol Hexakisphosphate

InsP6 is contained mainly in cereals, legumes, and oilseed [15]. The presence of a phosphate group in positions 1, 2, and 3 (axial-equatorial axis) confers unique properties to it as this configuration provides a specific chelating capacity regarding polyvalent cations, including iron and other potentially toxic elements (Ni, Zn, Cu, and even Uranium) [16, 17]. This property makes InsP6 an excellent chelator of many potentially harmful trace elements that have been shown to cause deleterious effects in humans [18]. Moreover, InsP6 capacity in blocking hydroxyl radical formation makes phytic acid a strong physiological antioxidant [19]. Insofar as InsP6 is often referred to as an antinutrient [20] responsible for iron deficiencies mostly in underdeveloped countries, it should be emphasized that InsP6 displays its antinutritional effects only when the diet is already deprived of trace elements [21].

Dietary InsP6 is mainly digested in the gut by bacterial phytases and phosphatase [22], thus releasing myo-Ins and other inositol phosphates (InsPs). Yet, a variable fraction of dietary IP6 is directly absorbed as such and can be recovered in plasma and urine [23], even if this assumption has been subject of controversy [24, 25].

A mixed western diet provides the human adult with approximately 1 g of total inositol per day [26]. No requirement for dietary inositol in man has been determined, even if physiological needs could be highly variable depending on the person's age, the long-term use of antibiotics, or the regular consumption of coffee [27]. How appropriate the bioavailability of myo-Ins and InsP6 in western alimentary regimens is still constitutes a matter of debate [28, 29]. By considering that from the '70s many foods have been processed to remove phytic acid (owing to its alleged antinutritional effects) it may be surmised that in western countries low-vegetable consumers may suffer from a relative deficiency of myo-Ins due to the reduced content of both myo-Ins and phytic acid in the diet. Furthermore, assessment of myo-Ins requirements is further complicated by the fact that a significant amount of inositol is endogenously synthesized from glucose. Glucose-6-phosphate is isomerized by D-3-myo-inositol-phosphate synthase (MIPS1 encoded by the ISYNA1 gene) to yield inositol-3-phosphate (Ins3P) and then converted by inositol monophosphatase-1 (IMPA-1) into free myo-Ins [30]. Both enzymes are inducible in response to the specific tissue requirements, thus explaining why myo-Ins concentrations differ so greatly among different tissues and physiological conditions [31].

After cellular internalization through endocytosis InsP6 is partially dephosphorylated yielding myo-inositol and inositol phosphates, mainly InsP5 [32]. However, free myo-Ins is actively transported into cells by a means of complex transport system. Within cells myo-Ins is converted into inositol phospholipids (phosphatidylinositol, PI, and phosphatidylinositol phosphate(s), $PIP_{(s)}$), inositol-glycans (IPGs), inositol phosphates (InsPs, including InsP6), and pyrophosphates (PP-IPs), according to a complex network extensively reviewed elsewhere [33, 34].

4. Molecular Mechanisms of Action

4.1. Cell Cycle Control and Apoptosis. Several studies have investigated the inhibitory activity of InsP6 on cancer cells from both animals and humans. Results are unambiguous and show that InsP6 induces G_1 phase arrest and abridges S phase of cancer cells, mainly by modulation of cyclins, upregulation of p53, p57, $p27^{Kip1}$, and $p21^{WAF/CIP1}$, and downregulation of phosphorylated pRb [35–37]. The family of pRB subunits (pRB/p107 and pRB2/p130) inhibits the cell cycle progression by forming complexes with E2F in the G0 phase. InsP6, by increasing the hypophosphorylated form of pRB, increases the pRB/E2F complexes formation, thus blocking further progression along the cell cycle [38]. Consequently, in InsP6-treated cancer cells, a significant downregulation of genes involved in cell cycle advancement (like c-*myc*, cyclin H, and FUSE) and an upregulation of those activated during cycle inhibition (CSK2, p57, and Id-2) have been observed [38]. Inhibition of breast cancer cell proliferation occurs independently from the estrogen receptor (ER) status, as it was achieved in both ER negative (MDA-MB-231) and positive (MCF-7) cells [39]. Similar results have been obtained in other cancers, even though subtle differences have been recorded among different cell lines [40, 41]. For instance, when leukemia cells were treated with InsP6, only some cell lines (A230 and K562) were arrested in G_2/M phase, while other cell lines (including $CD34^+$ from myelogenous leukemia patients) were committed to apoptosis [42]. Early studies have suggested that InsP6 effect is rather cytostatic than cytotoxic [43, 44]. However, further investigation demonstrated that InsP6 had unequivocal apoptotic effects on both solid and haematogenous tumors. Indeed, InsP6 has been shown to trigger programmed cell death both *in vitro* and *in vivo*

[45] in numerous cancer cell lines including Kaposi's sarcoma [46] and prostate, breast, cervical, pancreas, melanoma [47], and colon cancer [48–50]. This apoptotic effect is frequently associated with growth inhibition [35, 51] and ascertaining whether both effects occur independently from each other still needs to be investigated. Additionally, InsP6 has been shown to synergize with both doxorubicin and tamoxifen in inhibiting breast cancer growth, namely, in drug-resistant cancer cell lines [52]. This result implies that InsP6 may counteract drug resistance frequently displayed by tumor cells and should therefore be considered a useful adjunct in delivering conventional anticancer drugs. On the contrary, myo-Ins has been shown to have only a minimal proapoptotic activity and to induce a mild decrease in growth proliferation in colon, breast, soft tissue, and lung tumors [53]. Yet, myo-Ins is able to significantly synergize with InsP6, both *in vitro* and *in vivo*, in inducing cancer inhibition [54].

However, some results hint at a more subtle and complex role for inositol and its phosphate derivatives. In some circumstances, instead of apoptosis or growth inhibition, cell differentiation occurs after InsP6 treatment. Induction of differentiation in human erythroleukemia cells was preliminarily evidenced following InsP6 and subsequently in several other cancers, including rhabdomyosarcoma and breast, colon, and prostate tumors [55–57]. Why cancer cells respond so differently following InsP6 administration is poorly understood. It can be hypothesized that other factors, namely, other inositol phosphate derivatives, may participate in such processes, thereby driving the final output into diverse fates [58]. Yet, the contribution of context-dependent cues in modulating InsP6 effects cannot be discarded.

4.2. The p53 Network. Inhibition of cell proliferation and induction of apoptosis have been recorded in numerous cancer cell lines after InsP6 treatment. A crucial factor in both issues is represented by p53 activity and the subsequent selective pathways triggered downstream of p53. InsP6 increases p53 levels severalfold at both mRNA and protein levels [47, 59]. However, consistent data suggest that p53 is not mandatory for triggering InsP6-related effects, as apoptosis and inhibition of cell growth have been both observed in cancer cells lacking p53 [60]. On the contrary, p27 and p21 should be considered as essential molecular target of InsP6, given that the simultaneous knockdown of both p21 and p27 completely abrogates the anticancer effects of InsP6 [51]. By analogy, myo-Ins has been proven to reduce lung cancer incidence in mouse lacking p53 and treated with N-nitrosomethylurea [61]. Yet, a very recent paper demonstrated that oral myo-Ins does not suppress cancer development in p53 knockout mice [62], while evidence about the proapoptotic effect of myo-inositol is still inconclusive even in presence of p53. Thereby the question is still open and further studies are warranted to understand whether p53 activity is effectively required in mediating anticancer effects displayed by both InsP6 and myo-Ins. Downstream of p53 InsP6 has been demonstrated to reduce prosurvival factors and to upregulate caspases and other components of the proapoptotic BCL-2 family [63–66]. Furthermore, InsP6 has been shown to inhibit NF-kB activity in different cancers [67, 68]. NF-kB is a pivotal factor involved in fostering both survival pathways and the epithelial-mesenchymal transition (EMT). Therefore, targeting NF-kB is currently deemed a promising approach in cancer management. In prostate carcinoma, constitutive activation of NF-kB is inhibited by InsP6 [69], while in HeLa cells phytic acid prevents nuclear translocation of NF-kB and NF-kB-luciferase transcription activity [49]. In Caco-2 colon cancer cells, InsP6-mediated NF-kB inhibition is likely to occur through the block of the p65 subunit of NF-kB and its inhibitor IkBa [50].

As observed with other natural compounds (grape seed extracts, melatonin), the apoptotic effect triggered by inositol derivatives seems to be specific for cancer cells, given that both InsP6 and myo-Ins did not promote apoptosis in normal cells. Moreover, a "paradoxical" antiapoptotic effect of InsP6 has been noticed in normal cells exposed to iron-induced apoptosis [70]. Therefore, why normal and cancerous cells respond differently to both InsP6 and myo-inositol still deserves to be explained in detail.

4.3. Inhibition of the PI3K/Akt Pathway. The PI3K/Akt pathway is undoubtedly a pivotal hub upstream the activation of survival pathways, including the activation of Wnt and NF-kB [71]. PI3K triggers activation of Akt kinases through direct binding to the pleckstrin homology domain and the subsequent phosphorylation of Akt at two conserved residues. Hence, activated Akt modulates the function of numerous substrates involved in the regulation of cell survival, cell cycle progression, and cellular growth, eventually enabling cancer cells to become more aggressive [72]. These findings make the PI3K/Akt pathway one of the most attractive targets for therapeutic intervention. It is therefore worth noting that both InsP6 and myo-Ins significantly reduce PI3K expression (at both mRNA and protein levels) [73] and Akt activation by inhibiting its phosphorylation [74, 75]. InsP6 impairs directly PI3K activity and thus the PI3K-dependent activation of the tumor promoter-induced AP-1, as well as the phosphorylation-dependent activation of ERK [75]. Inhibition of PI3K activity and subsequent blocking of PKC and mitogen-activated kinases (MAPK) have been so far documented by several *in vitro* [76–78] and *in vivo* chemopreventive studies [79, 80]. Additionally, InsP6 interacts with clathrin-associated protein complex-2 and inhibits PI3K, ERK, and MAPK activation, thus impairing ErbB1 endocytosis and ligand-induced Shc phosphorylation [81]. Given that PI3K/Akt pathway activity is mandatorily required for triggering EMT, blocking PI3K would hinder the transformation of cancer cells into a more aggressive phenotype. Indeed, breast cancer cells treated *in vitro* with myo-Ins showed increased E-cadherin, downregulation of metalloproteinase-9, and redistribution of β-catenin behind cell membrane, while motility and invading capacity were severely inhibited [75]. Those changes were associated with a significant downregulation of PI3K/Akt activity, leading to a decrease in downstream signaling effectors: NF-kB, COX-2, and SNAI1. Moreover, myo-Ins decreases presenilin-1 (PS1) levels and inhibits its activity, thus leading to lowered Notch-1 release and SNAI1 levels. Furthermore, inositol-treated cells underwent profound cytoskeleton remodeling [75]. Overall,

these data indicated that myo-Ins inhibits the principal molecular pathway supporting EMT in cancer cells.

4.4. Inhibition of Invasiveness and Motility. The ability of cancer to metastasize relies primarily on the invasiveness and increased motility of tumor cells. It is therefore worth noting that, by blocking EMT, myo-Ins significantly hampers both motility and invasiveness of breast cancer cells. This effect is likely to be ascribed to cytoskeleton remodeling and to the concomitant inhibition of metalloproteinases (MMPs) release [75]. Similarly, InsP6 significantly reduces the number of lung metastatic colonies in a mouse metastatic tumor model [82], while in MDA-MB-231 breast cancer cells this effect is mediated by reduced adhesion and MMPs release [83, 84].

4.5. Wnt Signaling and Anti-Inflammatory Effects. Activation of the Wnt/β-catenin pathway occurs in several cancers. Overexpression of the Wnt ligand, usually in association with deregulated γ-secretase activity, may lead to deregulated expression and redistribution of β-catenin and of several molecular factors belonging to the so-called inflammatory pathway, like COX-2 and PGE2 [85]. Increased expression of the aforementioned molecules has been demonstrated to be associated with carcinogenesis in numerous tissues, chiefly in colon cancer [86]. It is of high relevance that InsP6 downregulates both *in vitro* and *in vivo* the Wnt pathway *via* β-catenin inhibition, thus significantly reducing COX-2 at both the mRNA and protein levels [87]. Eventually, this study demonstrated that InsP6 administration markedly suppressed in a dose-dependent manner the incidence of cancer in male Sprague Dawley rats when compared to controls. Moreover, InsP6 counteracts the proliferative response following inflammatory injury by inhibiting cyclin D1 and histone H3 expression [88].

In breast cancer cells, myo-Ins has been proven to downregulate both NF-kB and COX-2, while relocating β-catenin behind cell membrane [76]. Such inhibitory effects on inflammatory markers may not be confined to epithelial cells but should also probably involve the surrounding microenvironment. Indeed, both InsP6 and myo-Ins have been demonstrated to prevent pulmonary fibrosis, breast density, and chronic inflammatory damage, likely by influencing the crosstalk among cells and their milieu [89–91]. Given that TGFβ-1 released by both fibroblasts and epithelial cells is a profibrogenic factor regulating the balance between matrix-degrading metalloproteinases and their inhibitors [92], it is quite exciting that myo-Ins has been demonstrated to modulate the expression of both TGFβ and its receptors. Indeed, myo-Ins mitigates colonic epithelium inflammation as well as inflammatory consequences on colon stromal cells during microbial infections [93, 94]. Furthermore, InsP6 has been shown to exert valuable effects on fibroblasts by blocking the syndecan-4 dependent focal adhesion and microfilament bound [95]. Syndecan-4 is a heparan sulphate proteoglycan embedded into cellular membranes, where it regulates cell-matrix interactions by interfering with cytoskeleton proteins and integrins. Indeed, in human mammary cancer cell lines, cell adhesion to extracellular matrix was decreased after InsP6 treatment [84]. Moreover, syndecan binds to the fibroblast growth factor (FGF), fostering its coupling with the FGF receptor. InsP6 disrupts such interaction, thus inhibiting the FGF-based signaling [96]. Inositol-related effects on the cell *milieu* also involve modulation of angiogenesis. Formation of new blood vessels is required for sustaining cancer growth and invasiveness. Disruption of the structural relationships among cancer cells and their microenvironment promotes neoangiogenesis, mainly through the release of vascular endothelial growth factor (VEGF). InsP6 negatively modulates VEGF release from tumor cells [45] and impairs endothelial cells growth [97]. Likely, VEGF reduced synthesis may be due to InsP6-mediated inhibition on PI3K/Akt and MAPK/ERK pathways [82], given that both of them are deemed to modulate VEGF upregulation [98, 99]. Additionally, the synergistic activity of hypoxia and IGF-II increases VEGF mRNA expression and upregulates HIG-1 protein that, in turn, reinforces VEGF release [100]. Given that InsP6 has been shown to antagonize IGF-II activity by inhibiting the IGF-II receptor binding [101], it is likely that some InsP6 antiangiogenic effects can be ascribed to this mechanism.

Overall, these data suggest that inositol and its phosphate derivatives exert complex biological functions involving both cells and stromal factors. Yet, given the entrenched correlations occurring among cells and microenvironment during carcinogenesis [102, 103] the stromal effects of both InsP6 and myo-Ins deserve to be still fully investigated.

4.6. Anticancer Activity through Insulin Modulation. Myo-inositol and its isomer D-chiro-inositol (D-chiro-Ins) participate in both insulin and glucose metabolisms, and deregulated myo-Ins metabolism has been documented in several conditions associated with diabetes or insulin resistance [3]. Indeed, low levels of inositol have been observed in biological fluids and insulin target tissues (muscle, liver, and fat), frequently associated with excessive myo-Ins renal excretion, while low intracellular levels of myo-Ins have been detected in insulin insensitive tissues [104]. When insulin binds to its receptor, two distinct inositol-phosphoglycans (IPGs), incorporating either myo-Ins or D-Chiro-Ins (IPG-A and IPG-P), are released by insulin-stimulated hydrolysis of glycosyl-phosphatidylinositol lipids located on the outer leaflet of the cell membrane. IPGs affect intracellular metabolic processes, namely, by activating key enzymes controlling the oxidative and nonoxidative metabolism of glucose and acting as insulin-mimetic when administered *in vivo* in normal or diabetic rats [105]. Glycan derivatives of inositol significantly reduce insulin resistance and promote appropriate glucose metabolism [106]. Given that myo-Ins may efficiently counteract insulin resistance and its metabolic complications [107], it is tempting to speculate that it may also prevent IGF-1 increase associated with insulin resistance. As both insulin resistance and IGF-1 are linked to increased cancer risk [108], it is conceivable that myo-Ins modulation of insulin activity may efficiently contribute to reducing cancer risk. Indeed, InsP6 has been already shown to inhibit the IGF-1 receptor pathway-mediated sustained growth in cancer cells [85]. Moreover, cancer cells are featured by a glycolytic metabolomic fingerprint, thought to confer a

"proliferative advantage" during the neoplastic development [109]. It is therefore tempting to speculate if inositol addition can antagonize cancer development by normalizing glucose metabolism in cancer cells, another matter that eventually still needs to be fully investigated.

4.7. Antioxidant and Other Effects. Myo-Ins displays a moderate antioxidant activity, while InsP6 is among the strongest antioxidants present in nature. By chelating polyvalent cations, InsP6 and myo-Ins suppress Fenton's reaction and the consequent release of hydroxyl radicals [110]. In biological tissues InsP6 has been shown to inhibit xanthine oxidase [111] and reactive oxygen species production, thus dramatically inhibiting the free radical-based damage occurring in cells and tissues following inflammation, hypoxia, or exposition to radiation injury [91, 112, 113]. Myo-Ins counteracts oxidative damage in fish exposed to environmental stresses [114] and significantly inhibits systemic markers of oxidative stress in gynecological patients [115]. InsP6 scavenges superoxide radicals *in vitro* and *in vivo*, thus preventing formation of ADP-iron-oxygen complexes that trigger lipid peroxidation [116]. Indeed, inhibition of lipid peroxidation has been documented in animals after InsP6 administration [117, 118]. As increases in both ROS and lipid peroxidation have been associated with cancer development, it has been hypothesized that some anticancer chemopreventive effects displayed by InsP6 and myo-Ins could therefore be ascribed to their antioxidant capability. However, as recorded for other natural compounds, the antioxidant property of inositol is strictly context-dependent as, under specific conditions, both myo-Ins and InsP6 may increase free radical production [119].

5. Effects on the Immune Function

Even if it is still limited, current evidence suggests that inositols may play an appreciable regulatory activity on immune function *in vitro* and *in vivo*. Inositol hexakisphosphate and myo-Ins enhance NK activity in mice treated with 1,2-dimethylhydrazine (DMH), a colon carcinogen, which also significantly reduces NK function [120, 121]. In this model, InsP6 also reverses tumor induction, decreases cancer-related death, and specifically boosts NK cytotoxicity in a dose-dependent manner. As previously observed in other studies, the association of InsP6 and myo-Ins displays synergistic effects, given that significantly better results were observed in animals treated with a combination of both [120]. InsP6 acts as a neutrophil priming agent and it upregulates several neutrophil functions, including enhancing superoxide production and phagocytosis [119]. Additionally, InsP6 modulates a number of inflammatory markers, namely, involving IL-8 release by stimulated neutrophils [119]. InsP6 also modulates the transcription genes for TNF by decreasing it and its receptors in colon cancer cells [122]. This downregulating effect of InsP6 on inflammatory processes is mirrored by the aforementioned inhibitory activity displayed by myo-Ins on several inflammatory pathways (COX-2 and PGE2) [76]. Therefore, it seems that both inositols exert inhibitory control on the activation of the inflammatory pathways, which are frequently upregulated during carcinogenesis.

6. Chemopreventive and Therapeutic Efficacy in Animal Studies

The chemopreventive as well as the therapeutic activity *in vivo* of InsP6 has been documented by an impressive body of studies. Exogenous administration of InsP6 in drinking water, one or two weeks after azoxymethane induced carcinogenesis, prevents the onset of colon cancer in Fisher rats [123]. Preventive activity was also observed when InsP6 was added in higher concentration 5 months later after the carcinogenic stimulation [124]. Inositol hexakisphosphate can indeed prevent even the formation of aberrant colon crypts, thought to be the histological precursor of the neoplastic transformation [125].

On the other hand, inositol hexakisphosphate may potentiate the anticancer effects of conventional chemotherapy in preventing the successful development of cancer implants. Indeed, the administration of liposomes containing both InsP6 and irinotecan (CPT-11) showed higher efficacy in inhibiting the viability and the growth of colon tumor xenografts in mouse when compared to single compounds alone [126]. InsP6 chemopreventive activity is not restricted to the gastrointestinal tract, as it has been shown that inositols may efficiently counteract the carcinogenic effect of chemicals on breast tissue. Breast tumor incidence after exposure to 7,12-dimethylbenz[*a*]anthracene or N-methylnitrosourea is significantly reduced in animals treated with InsP6. Similarly, InsP6 dramatically reduces by almost 64% the burden of implanted DU-145 prostate cancer [35] as well as the growth of transgenic adenocarcinoma of the prostate in mouse [127, 128]. Similarly, inositol hexakisphosphate inhibits growth and induces G_1 arrest and apoptotic death of androgen-dependent human prostate carcinoma LNCaP cells [40]. Namely, in a transgenic mouse model of prostate carcinoma (TRAMP), orally administered InsP6 has been able to inhibit cancer progression at prostatic intraepithelial neoplasia stage and strongly reduced the incidence of adenocarcinoma (prostatic intraepithelial neoplasia/adenocarcinoma, 75 : 25% in the InsP6 group versus 39 : 61% in the control group) [127]. These findings evidence the chemopreventive efficacy of oral administered InsP6 *in vivo* as well as its safety.

Additionally, InsP6 or myo-Ins has been shown to induce the regression of other different types of cancer, like rhabdomyosarcoma, liver cancer [129], soft tissue [130], and fibrosarcoma [43, 83, 131]. Skin tumorigenesis induced by chemical compounds [44] or by physical factors (i.e., UVB) [68] was also demonstrated to be significantly diminished by InsP6 administration. Both InsP6 and myo-Ins reduced the incidence and growth of lung tumors chemically induced in mice [132]. Indeed, dietary inositol has been shown to inhibit lung tumorigenesis in female A/J mice exposed to the carcinogen benzo(α)-pyrene or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone: myo-Ins was also effective in the postinitiation phase and when given for short periods of time before, during, and immediately after carcinogen exposure [133, 134]. Overall, myo-Ins anticancer efficacy was proven to be lower when compared to InsP6. However, it is noticeable that myo-Ins potentiates significantly the antitumor effects displayed by InsP6 *in vivo* [135, 136].

7. Chemopreventive and Therapeutic Efficacy in Human Clinical Trials

Both myo-Ins and InsP6 are safe, even when administered at high doses, as assessed by several clinical trials performed in cancer patients as well as in humans suffering from other diseases (mostly with gynecological diseases like PCOS). Mild side effects (mostly represented by nausea or diarrhea) are reported in a small fraction of subjects, only for doses up to 12 g/day (reviewed in [137]).

On the other hand, InsP6 has been demonstrated to exert valuable anticancer effects even *in vivo* when administered to cancer patients. An antitumor activity has been observed in advanced colon cancer patients, where InsP6 plus myo-Ins treatment is associated with appreciable reduction in tumor burden and improved quality of life. Moreover, if inositols were added along with conventional chemotherapy, colon cancer patients experienced significantly less side effects than controls, as reported in a pilot study [138]. Furthermore, prolonged survival and better quality of life have been obtained in some anecdotal cases of breast and lung cancer patients treated with InsP6 and myo-Ins [139–141]. Again, in a prospective, randomized study, InsP6 and myo-Ins ameliorate the responsiveness to chemotherapy in breast cancer patients and markedly reduce the burden of side effects [142]. As previously noticed in animal studies, myo-Ins has been demonstrated to exert a significant chemopreventive activity also in human beings [143]. A study enrolling 26 smokers showed that myo-Ins in a daily dose up to 18 g/p.o. is safe and well tolerated, while inducing a significant regression of individual pulmonary dysplastic lesions (91% in the inositol-treated group versus 48% in control group) in a sample of heavy smoker individuals [144]. A significant increase in a genomic signature of PI3K pathway activation has been documented in the cells of the bronchial airway of patients with dysplastic lesions, thus suggesting that PI3K is activated in the proximal airway before tumorigenesis. Treatment with myo-Ins is able to induce a marked regression of both dysplastic lesions and PI3K activity. Such preliminary findings have been subsequently established by two other papers [79, 80]. Unfortunately, as these trials have been carried out on very small samples of patients, no firm conclusions can be drawn from them.

Overall, those data represent, at best, only a promising preliminary hint, seldom emerging from anecdotal observations. Indeed, a number of critical factors actually limit the clinical relevance of the available results.

First, no extensive, randomized trials have been done till now. Pilot studies are potentially flawed by the reduced number of enrolled patients and (with some exceptions) the lack of randomization. Well-designed clinical studies are thereby required to evaluate, if any, the different responsiveness among men/women and the diverse sensitivity of solid/hematological cancers when treated with inositol(s). Due to the hypothetical mechanisms of inositol action, these surveys would require an extended period of observation and larger patient samples than those studied till now. That remark applies also to chemopreventive studies. Even if there are no ongoing or planned randomized clinical trials with either InsP6 or myo-Ins, a recent clinical study promoted by the NIH [145] showed no benefit associated with myo-Ins supplementation in heavy smokers carrying bronchial dysplasia. Yet, even this survey is biased by the limited number of subjects (38 in the myo-Ins arm versus 36 placebo-treated controls) entering the study.

Second, clinical studies should be aimed at recording not only the response in terms of cancer changes but also the concomitant modification in metabolic/endocrine milieu. Indeed, an almost entirely overlooked field of investigation is represented by the involvement of inositol(s) in estrogen modulation. This is a potentially outstanding issue, as inositol(s) have been shown to modulate aromatase activity as well as a number of circulating hormones (including insulin, FSH, and LH). Studies performed in women affected by PCOS have shown that aromatase activity and the release of gonadotropin-releasing factors (LH and FSH) and of numerous other hormones (including insulin, prolactin, and testosterone) are significantly modulated by inositol addition [146]. It is therefore tempting to speculate that such mechanism may also participate in triggering inositol-related anticancer effects on endocrine responsive tumors (especially breast and prostate cancer).

Third, clinical benefit ensured by the addition of InsP6 and/or myo-Ins to conventional chemotherapy may be ascribed to indirect physiological effects rather than to a "direct" anticancer effect. As previously outlined, both InsP6 and myo-Ins modulate a number of proinflammatory pathways by targeting few components of cancer stroma (fibroblasts and density of the surrounding matrix). Modulation of cancer stroma, in association with the inositol-based effect on the cytoskeleton, may efficiently contribute to reframing the functional architecture of cancer microenvironment [103], thus leading to a plethora of unexpected consequences, ultimately ending up into an inhibition of cancer growth.

Inositol(s) may indeed modulate the antioxidant/prooxidant balance, as well as the patient metabolomic fingerprint (downregulation of insulin levels, improved glucose utilization through the oxidative cycle, and inhibition of lipogenesis). Such effects have been extensively recorded in nonneoplastic patients suffering from PCOS or metabolic diseases [3, 147] and likely may also be effective in cancer patients.

8. Outstanding Issues

Both myo-Ins and InsP6 have been demonstrated to exert a wide range of anticancer effects. Namely, inositols interact with specific cancer cellular pathways, while also exerting other valuable activities at the systemic level (enhancement of immune function, antioxidant activity). The astonishing complexity of their effects (Figure 1) on so different targets allows us to consider both of them as truly "pleiotropic" agents. Moreover, as suggested by some preliminary reports, it cannot be discarded that InsP6 and myo-Ins may also play a specific epigenetic role in selected gene clusters.

8.1. Epigenetic Effects. In yeast, myo-Ins displays basically a repressing activity on a discrete number of genes [148], and preliminary data suggest that this is also the case in humans

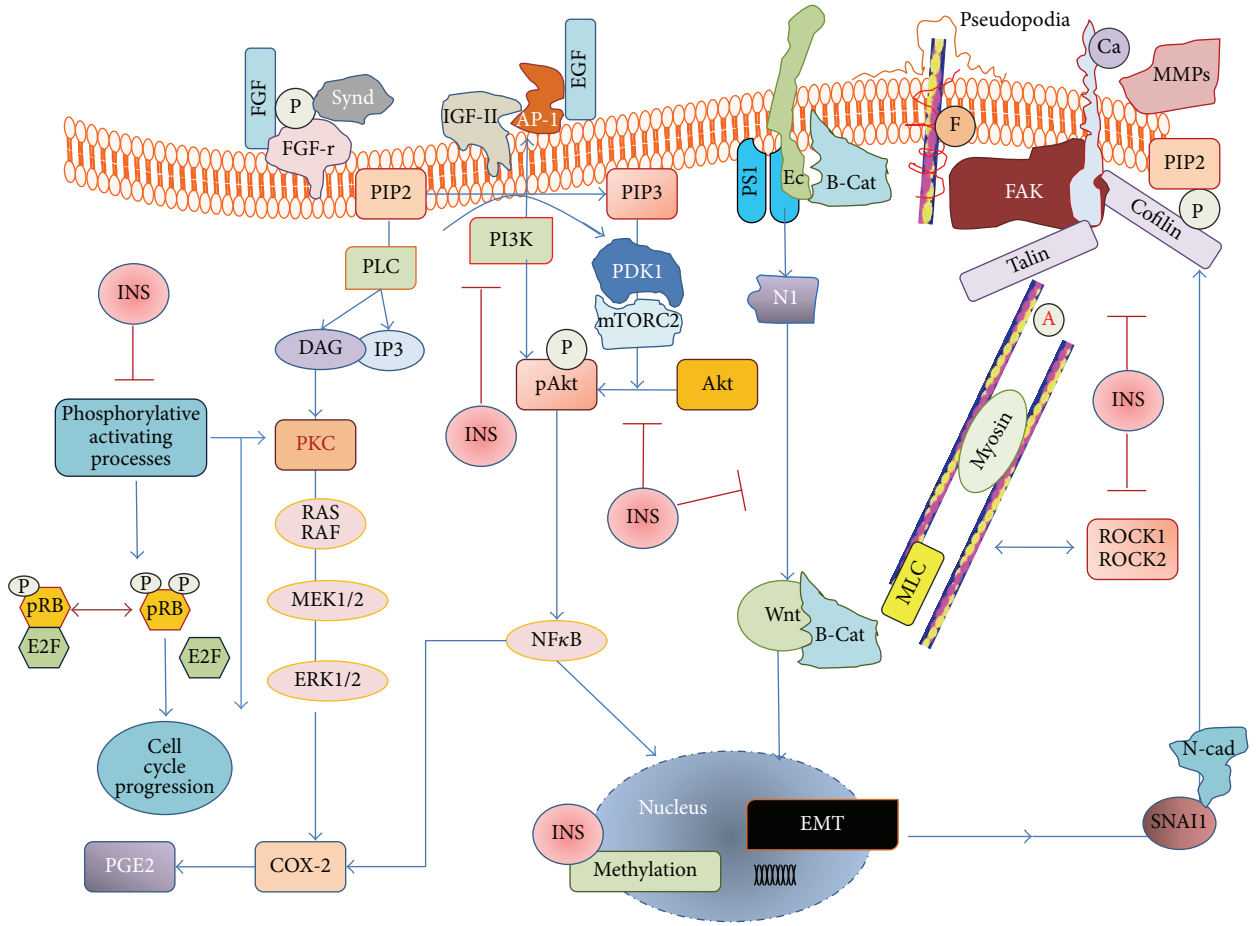

Figure 1: Inositol mechanisms of action in cancer cells. Inositols (INS), including InsP6 and myo-Ins, modulate a number of different critical pathways. Available data suggest that the inhibition of the phosphorylation-based (P) activation of key molecular targets represents a basic mechanism through which inositol interferes with specific biological functions, eventually ending up in delaying cell replication and in fostering apoptosis or phenotypic differentiation. (A) Inositols inhibit pRB phosphorylation, thus fostering the pRB/E2F complexes formation and blocking further progression along the cell cycle. (B) Phosphatidylinositol-4,5-bisphosphonate (PIP2) is metabolized to diacylglycerol (DAG) and Ins-trisphosphate (IP3) by phospholipase-C (PLC). Moreover, PI3K catalyzes the synthesis of PIP3 from PIP2. PIP3 is required for enabling the activation of ERK and Akt pathways. Indeed, by reducing both PI3K levels and its activity, inositols counteract the activation of the PKC/RAS/ERK pathway. Upstream of that pathway, inositols disrupt the ligand interaction between FGF and its receptor (FGF-r) by interfering with syndecan (Synd) activity as well as with the EGF-transduction processes involving IGF-II receptor and AP-1 complexes. Downstream of PI3K inhibition, Akt activation through selective phosphorylation promoted by PDK and mTORC2 is severely impaired upon inositol addition. Downregulation of both Akt and ERK leads consequently to NF-kB inhibition and reduced expression of inflammatory markers, like COX-2 and PGE2. Inositol-induced downregulation of presenilin-1 (PS1), when associated with inhibition of the PI3K/Akt pathway, counteracts the epithelial-mesenchymal transition (EMT), thus reducing Wnt-activation, β-catenin (β-cat) translocation, Notch-1, N-cadherin (N-cad), and SNAI1 release. Inositols interfere also directly with different cytoskeleton components by upregulating Focal Adhesion Kinase (FAK) and E-cadherin (Ec) and decreasing Fascin (F) and Cofilin, two main components of the pseudopodia. Reduced formation of membrane ruffling and pseudopodia, as well as inhibited release of metalloproteinases (MMPs), severely impairs both motility and invasiveness of cancer cells. This effect is reinforced by the inositol-induced inhibition on ROCK1/2 release, as well as by the decreased levels of phosphorylated Myosin Light Chain (MLC). Overall, these effects enable inositols to remodel F-actin (A) assembly and thus to reshape the cytoskeleton architecture. Blue arrow indicates promoting effect; red line with bar indicates inhibitory effect.

(personal communication). Conversely, under conditions of inositol deprivation, hundreds of genes are activated, mainly those involved in stress response pathways [149], PKC pathways [150], inositol and phospholipid biosynthesis (ISYNA1 gene) [151, 152], and glucose metabolism [153]. It is still to be investigated if the inositol-based control on gene expression should be ascribed to methylation specific activity or to other mechanisms. In addition, myo-Ins has been shown to be involved in chromatin remodeling and DNA-repair processes. Chromatin remodeling represents a critical process ruling the access for DNA-binding proteins and therefore it is required for efficient gene transcription. Mutation in genes encoding inositol polyphosphate kinases responsible for the production of InsP4, InsP5, and InsP6

impairs gene transcription *in vivo*, thus evidencing that specific inositol phosphates are required for proper transcriptional activity, thus establishing a clear link between InsPs availability and chromatin remodeling [154, 155]. Indeed, inositol phosphates are involved in gene transduction, given that depletion of InsP6, InsP7, and InsP8 by means of inositol polyphosphate multikinase inhibition impairs mRNA export from the nucleus [156]. Efficiency of gene transcription relies on DNA stability and maintenance that is primarily ensured by DNA-repair mechanisms. Homologous recombination and nonhomologous end-joining are the two main DNA-repair mechanisms frequently deregulated in a number of pathological conditions. Inositol phosphates (mainly InsP6) have been shown to foster DNA-repair processes by binding to the DNA end binding protein Ku [157]. Inositol hexakisphosphate modulates Ku dynamics [158] by interacting with a specific Ku region and, by subsequently activating the DNA-PK binding, InsP6 promotes the nonhomologous end-joining repair [159]. Furthermore, it has been shown that InsP6 binds to DNA-PK and specifically stimulates DNA-PK-dependent end-joining *in vitro* [158].

8.2. Synergistic Effects. There is a widespread consensus suggesting that InsP6 and myo-Ins act synergistically when added in association. That finding evidences a possible cumulative effect on selected targets or, even more likely, a complex metabolic interaction. InsP6 has indeed been demonstrated to be dephosphorylated within the cell, leading to myo-Ins or to less phosphorylated forms (namely, InsP5 and InsP4) [23, 160] which, in association with myo-Ins, may collectively modify the network of inositol-based molecules and hence a number of biochemical pathways. Moreover, a number of inositol derivatives, including lower phosphorylated forms [161, 162] and pyrophosphates [163], have been proven to exert anticancer effects. However, despite the fact that some insight has been provided by using [^{3}H]InsP6 [164] or [^{3}H]myo-Ins [165], we are still unable to grasp what the cellular fate of both InsP6 and myo-Ins could be after the cellular uptake. Additionally, inositol isomers may also play a significant biological role, hitherto evidenced in other diseases. For example, the association of myo-Ins and D-chiro-inositol in a proper ratio (40 : 1) has been demonstrated to be effective in polycystic ovary syndrome treatment [166], while *scyllo*-Ins is currently under scrutiny as a reliable treatment for Alzheimer and other neurological diseases [167]. It would be worth of interest to ascertain whether inositol isomers or other inositol derivatives could also exert any valuable biological effect in cancer. It is therefore mandatory to investigate thoroughly the inositol metabolomics in order to identify the main metabolic pathways of both InsP6 and myo-Ins. Furthermore, metabolomics data should be integrated with genomic pathways, thus providing the basic information required to recognize the cellular fate of therapeutically added inositols and the genomic/enzymatic targets downstream.

8.3. Pleiotropic Effects. Inositol and its phosphorylated derivatives (InsP6 and InsP5) interfere with several critical processes involved in the regulation of cell proliferation, apoptosis, and differentiation, including the MAPK-ERK cascade, the PI3K/Akt, and the β-catenin/Wnt/NF-kB pathway. The PI3K/Akt pathway has been proven to be inhibited by a wide range of inositol phosphates (InsP6, InsP5, and InsP4) [168] as well as by myo-Ins. This effect can be ascribed to several mechanisms including direct PI3K blocking (as the structure of InsP6 appears to be very similar to 3-deoxy-3-fluoro-PtdIns, a potent PI3K inhibitor) [169] or inhibiting the PI(3,4,5)P3-dependent Akt recruitment to the plasma membrane [170]. Moreover, it seems that myo-Ins, InsP6, and other inositols phosphate derivatives may modulate cell function by inhibiting several phosphorylation pathways. Activation mechanisms through phosphorylation of Ras, mitogen-activated protein kinases (MAPK), protein kinase C (PKC), PI3K, and activating-protein-1 (AP-1) are indeed downregulated by inositols via a direct control of protein phosphorylation. InsP6 inhibits the phosphorylation-induced activation of ERK and JNK activity in a number of cancer types [75, 82, 171]. InsP6 selectively activates two distinct isoforms of PKC: PKC-ε and PKC-δ. PKC-ε is required for insulin secretion and primes Ca^{2+}-induced exocytosis in pancreatic β-cells upon InsP6 stimulation [172]. PKC-δ activity is increased severalfold after InsP6 addition, and that increase leads subsequently to enhanced release of p27, thus blocking cell cycle progression in breast cancer cells [36]. Phosphorylation of specific residues seems to be a widely used mechanism in nature for activating specific molecular effectors, while dephosphorylating performed by phosphatases (like PTEN [173], SHIP [174], or inositol polyphosphate phosphatases [175]) represents a general inhibitory tool for counteracting the same pathways. Therefore, the complexity of the inositol metabolism stands out in the midst of the even more complex field of enzymatic regulation and it is quite impossible to deal with this complexity only relying on the rules provided by the old-fashioned reductionist model. On the contrary, a systems biology approach [176] is mandatory to efficiently grasp the interwoven inositol network.

9. Conclusion

Myo-inositol and its derivatives, among which InsP6 occupies a relevant place, have been shown to play many biological functions, including modulation of cell cycle progression, apoptosis, and differentiation. During the last decade, evidence is mounting that inositol acts on both cytosolic and nuclear targets in enabling cells to successfully cope with many different stressors. Indeed, the inositol network seems to display a key role during developmental processes and cellular differentiation, as demonstrated by studies carried out on oocyte maturation and embryo development [177, 178].

Available results suggest that the combination InsP6+ myo-Ins may be most effective to move forward in the future. It can be hypothesized that this association may enact the release of low-phosphorylated inositol derivatives (InsP5, InsP4, InsP3, and InsP2), which in turn may trigger specific effects. Alternatively, InsP6 and myo-Ins may target the same molecular mechanisms or enzymatic pathway displaying true synergistic (rather than additive) effects. However, until a metabolomic profile of added myo-Ins will be available,

hypotheses on the synergistic effect of InsP6 and myo-Ins are at best presumptive.

Cancer can be considered a kind of "development gone awry" [179], in which the deregulation in the crosstalk among cells and their microenvironment plays a relevant role. Given that inositol participates in the cell-stroma interplay by modulating metalloproteinases, E-cadherin, focal kinase complexes, and many other cytoskeletal components, it can be hypothesized that inositol and its derivatives may counteract cancer-related processes by specifically acting at this level, that is, by restoring a "normal" cell-stroma relationship. Studies in this field are therefore urgently warranted in order to deepen our understanding of inositol mechanisms on cancer.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Review Article

Inositol Treatment and ART Outcomes in Women with PCOS

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Polycystic ovary syndrome (PCOS) affects 5–10% of women in reproductive age and is characterized by oligo/amenorrhea, androgen excess, insulin resistance, and typical polycystic ovarian morphology. It is the most common cause of infertility secondary to ovulatory dysfunction. The underlying etiology is still unknown but is believed to be multifactorial. Insulin-sensitizing compounds such as inositol, a B-complex vitamin, and its stereoisomers (myo-inositol and D-chiro-inositol) have been studied as an effective treatment of PCOS. Administration of inositol in PCOS has been shown to improve not only the metabolic and hormonal parameters but also ovarian function and the response to assisted-reproductive technology (ART). Accumulating evidence suggests that it is also capable of improving folliculogenesis and embryo quality and increasing the mature oocyte yield following ovarian stimulation for ART in women with PCOS. In the current review, we collate the evidence and summarize our current knowledge on ovarian stimulation and ART outcomes following inositol treatment in women with PCOS undergoing in vitro fertilization (IVF) and/or intracytoplasmic sperm injection (ICSI).

1. Introduction

Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder of women in reproductive age with a prevalence ranging from 5 to 10% [1]. According to the 2003 Rotterdam consensus workshop, it is defined by 2 out of the following 3 criteria: hyperandrogenism, oligo/anovulation, and polycystic ovarian morphology [2]. There is an increased risk of hypertension, cardiovascular disease, impaired glucose tolerance, type II diabetes, and dyslipidemia in women suffering from PCOS. Despite many decades of extensive research, the exact etiology of this disorder remains largely unknown, although it is recognized that ovarian hyperthecosis, increased androgens, insulin resistance, and genetic and environmental factors all play a role in the pathophysiology of this syndrome [3]. It is widely known that insulin resistance (IR) plays an important role in the pathogenesis of PCOS in a large subset of patients [4]. Insulin stimulates ovarian theca cells to produce and secrete androgens both directly and indirectly. Elevated glucose levels in turn inhibit the hepatic synthesis of sex hormone-binding globulin, leading to increased concentration of circulating free androgens [5]. Insulin resistance is a common feature in both overweight and lean women with PCOS independent of their body mass index (BMI) [6]. Approximately 80% of obese women with PCOS and 30–40% of lean women with PCOS have hyperinsulinemia due to IR [7]. As the relationships between IR and PCOS manifestations were established, several insulin-sensitizing compounds such as metformin and thiazolidinediones were demonstrated as effective adjuncts in treating PCOS women [8, 9]. In addition, over the last decade the use of myo-inositol, a natural insulin sensitizer, has been investigated in many studies of PCOS women. The role of myo-inositol in women with PCOS can be attributed to deficiency of metabolites of myo-inositol which are mediators of insulin action [10]. In addition to the beneficial effects of myo-inositol on insulin resistance and other metabolic aspects, it has been shown to improve oocyte maturation, follicular milieu, and outcomes of ART in women with PCOS [11, 12]. In this article, we review the effects of myo-inositol on ovulatory dysfunction in women with PCOS with a particular focus on the accumulating evidence associating myo-inositol with ART outcome in these women. Better understanding of

the role of myo-inositol in the reproductive function in PCOS may improve ART outcomes in women with PCOS.

2. Methods

A comprehensive literature search was performed using the electronic databases PubMed, Medline, and Google Scholar for relevant articles in the English language until May 2016. The following combinations of search terms were used: "polycystic ovarian syndrome", "PCOS", "inositol", "ovulatory dysfunction", "hyperandrogenism", "insulin resistance", "ovulation induction", "in vitro fertilization", "IVF", "Assisted reproductive technology", "ART", "metformin", and "treatment outcome". The abstracts identified were reviewed and evaluated by both authors. Any disagreement between the two researchers was resolved with discussion. The reference lists of the selected papers were manually searched in order to identify additional potentially relevant studies.

2.1. Inositols: Biochemistry and Sources. Inositols and their derivatives are sugar alcohols which belong to the vitamin B family, representing nine cyclohexane-1,2,3,4,5,6-hexol stereoisomers. They are chemically stable molecules which can be ingested from the diet [13]. Dietary products containing inositol include fruits, mainly cantaloupe and oranges, high bran content cereals, nuts, and beans. Fresh fruits and vegetable contain more inositol when compared with the frozen, salted, or canned products. The average daily intake of myo-inositol from the diet may vary between 225 and 1500 mg/day per 1800 kcal diet [14]. Inositols are not considered an essential nutrient since they can be formed endogenously from glucose [15]. Myo-inositol (MYO) and D-chiro-inositol (DCI) are the two out of nine existing stereoisomers of inositol that are formed after epimerization of the hydroxyl groups of inositol [12, 16–18]. Myo-inositol is the most abundant form of inositol in both nature and mammalian cells, comprising up to 99% of the inositol amount. The remaining 1% is represented by the other stereoisomer DCI. Endogenously, the synthesis of myo-inositol occurs from glucose-6-phosphate in the two-step process which involves conversion of glucose-6-phosphate to myo-inositol 1-phosphate by inositol-3-phosphate synthase enzyme, which is then dephosphorylated to form free myo-inositol by the enzyme inositol monophosphatase [19]. Myo-inositol is converted into DCI by an NAD/NADH epimerase. The activity of this insulin-dependent enzyme strongly influences the intracellular ratio between these two molecules in adipose, hepatic, or muscle cells [20]. Both of these molecules have been used as insulin-sensitizing agents in women with PCOS [21, 22]. Inositol is present in phospholipids, can stimulate lecithin production, serves as an important component of structural lipids, and also controls the fat and lipid metabolism.

2.2. Inositol and Reproductive Function. Inositols and their phosphates function as secondary messenger molecules for various cellular signaling pathways including insulin signal transduction, calcium trafficking, lipid metabolism, cytoskeletal protein assembly, cell growth and differentiation, modulation of serotoninergic pathways, oocyte maturation, and fertility [29]. Uptake of free inositol by tissues occurs by a membrane dependent sodium-inositol cotransporter [30]. However, in comparison to DCI, MYO has 10 times greater affinity for this cotransporter [31]. There is substantial evidence linking inositols to reproductive outcomes. Inositol has been demonstrated to play an important role in oocyte maturation and fertilization via its regulation of calcium signaling pathways [32, 33]. Inositol 1,4,5-trisphosphate receptor (IP3R) plays a key role in triggering the Ca^{2+} release during oocyte maturation and fertilization [34, 35]. Myo-inositol levels in blood and follicular fluid have been shown to positively correlate with oocyte quality and pregnancy outcome in humans [32, 36]. Moreover, supplementation of MYO has been suggested to promote meiotic progression of oocytes, producing better quality oocytes and embryos in mice [37, 38]. In humans, administration of MYO to women prior to hormonal stimulation in IVF cycles has been shown to increase oocyte and embryo quality and reduce the dose of FSH and the days required for stimulation [11, 39]. Furthermore, studies on PCOS women have shown that MYO can improve menstrual regularity, insulin resistance, and oocyte quality and maturation [23, 40, 41].

2.3. Inositol, Insulin Resistance, and Ovarian Function in PCOS. It is now recognized that insulin resistance has a critical role in the pathogenesis of PCOS. Hyperinsulinemia secondary to insulin resistance is common in a high proportion of PCOS patients and is associated with metabolic morbidities as well as reproductive dysfunction [42]. Insulin resistance is thought to contribute to hyperandrogenism in PCOS by insulin stimulating ovarian theca cells to produce and secrete androgens and by elevated glucose levels inhibiting the hepatic synthesis of sex hormone-binding globulin, leading to increased concentration of circulating free androgens [5]. While the exact cause of insulin resistance in PCOS is unknown, it has been postulated that a defect in the postreceptor transport of glucose and selective resistance to metabolic actions of insulin may be responsible for hyperinsulinemia in these patients [43, 44]. The well-known association between hyperinsulinemia, hyperandrogenism, and ovulatory dysfunction in PCOS formed a basis for treatment with insulin-sensitizing agents such as myo-inositol, metformin, and thiazolidinediones, which have proven effective in improving insulin resistance as well as ovarian functions in these women [45].

Inositol and its stereoisomers are classified as insulin sensitizers and act as a second messenger of insulin signaling pathways [46]. Indeed, the actions of insulin are mediated by two distinct inositol phosphoglycan (IPG) mediators, incorporating either MYO or DCI, which are released by the hydrolysis of glycosyl-phosphatidyl-inositol lipids on the outer side of the cell membrane. IPG in turn activates the key enzymes controlling the oxidative and nonoxidative glucose metabolism, affecting the intracellular metabolic processes. Both MYO- and DCI-containing IPGs decrease insulin resistance [47].

Women with PCOS have reduced serum level and increased urinary loss of DCI [48, 49]. It was demonstrated that DCI urinary clearance was inversely correlated with

insulin sensitivity in PCOS women and was a strong and independent predictor of insulin resistance in these women [48, 49]. Following these observations, it has been established that PCOS women have a dysregulation of inositol metabolism [50], providing a mechanistic link between inositol deficiency and insulin resistance in PCOS. Since IPG signaling pathways are involved in insulin-mediated thecal androgen biosynthesis, defective conversion of MYO to DCI in PCOS patients may also contribute to hyperandrogenism [51]. Indeed, administration of DCI at low doses has been shown to decrease insulin resistance and serum androgens and improve ovulatory frequency in PCOS women [16, 52, 53]. However, when administered at higher doses DCI appears to exert negative effects on the ovaries [54]. In fact, subsequent clinical trials performed with DCI doses of 2.4 g/day were unable to confirm previous positive results on PCOS women, suggesting that DCI may paradoxically worsen the ovarian response in these patients despite normalization of the IR parameters [53].

As mentioned earlier, MYO is converted to DCI via an insulin-induced epimerase enzyme in different tissues based on their requirement for each of these molecules [55, 56]. DCI conversion is reduced in muscle tissue of subjects suffering from insulin resistance due to decreased epimerase activity [20, 57, 58]. These studies included muscle and liver which can develop insulin insensitivity. However, in contrast to these tissues, normal ovaries and those of ovulatory PCOS remain insulin sensitive [59, 60]. Moreover, one study demonstrated higher M/C epimerase specific activity in theca cells of PCOS compared to control women and 4-fold higher MYO : DCI ratio in control than in PCOS [61]. Thus, hyperinsulinemia may alter the MYO : DCI ratio and paradoxically increase DCI concentration within the ovary. Decrease in MYO : DCI ratio has also been recently reported in follicular fluid of PCOS women [62]. These observations support the notion that any further increase in DCI is detrimental to ovarian function, explaining the lack of clinical benefit when using DCI in PCOS women and highlighting the importance of maintaining proper MYO : DCI ratio when administering inositols to PCOS women.

In contrast, the benefit of MYO supplementation in PCOS has been demonstrated by several studies. Administration of MYO combined with folic acid 2 g twice a day for 6 months in PCOS patients showed maintenance of normal ovulatory activity in 72%, with singleton pregnancy rate of 40% during the 6-month observation period [23]. In a recent study by Kamenov et al., 50 anovulatory PCOS patients with insulin resistance were given MYO for three spontaneous cycles and ovulation and pregnancy were achieved in 61.7% and 37.9% of women, respectively. In the women who remained anovulatory, MYO was used in combination with clomiphene citrate for three cycles resulting in ovulation and pregnancy rates of 72.2% and 42.6%, respectively. These patients also had reduction in BMI and HOMA index, suggesting the role of MYO-induced amelioration of insulin resistance, in mediating the improvement in ovarian function in women with PCOS [63]. While the above studies lacked a control group, similar beneficial effects on ovarian function were also noted by Artini et al. in their randomized controlled trial of 50 overweight PCOS patients which were divided into two groups; group A was given MYO 2 g plus folic acid 200 mg daily for 12 weeks and group B was given folic acid 200 mg daily. They found significant improvement in hormonal parameters and restoration of menstrual cyclicity in all amenorrheic and oligomenorrheic patients in group A while no changes were noted in group B, suggesting the role of MYO in improving the reproductive axis in PCOS patients [64]. Moreover, administration of both MYO plus DCI has been shown to be effective in achieving better clinical results in PCOS patients in a combination replicating the plasma physiological ratio (40 : 1) by working at systemic and ovary level [62, 65, 66]. The mechanism of the beneficial effects of MYO on ovarian function in the polycystic ovaries could be due to increase in glucose uptake and facilitating FSH signaling that likely improve oocyte quality and better IVF outcome [67].

Another important consideration when evaluating the effects of inositols in PCOS women is the interaction with obesity. Baillrgeon et al. showed that obese PCOS women have diminished release of DCI-IPG in response to insulin elevation compared to normal weight women [50]. Since this study did not include normal weight PCOS women, it is difficult to ascertain whether the observed abnormal DCI-IPG response was related to PCOS, obesity, or a combination of both. However, the investigators noted that reduced insulin-stimulated DCI-IPG response was significantly associated with obesity (BMI, $r = -0.56$, $P = 0.025$), suggesting that obesity plays a role in abnormal inositol metabolism independently of PCOS. In a randomized trial of inositol treatment in PCOS women, Gerli et al. have shown that metabolic risk factor benefits of inositol treatment were not observed in the morbidly obese subgroup of patients, noting an inverse relationship between BMI and treatment efficacy [12]. Recently, Ferrari et al. studied the effects of MYO/DCI supplementation on the maternal metabolic profile in mouse pregnancies complicated by obesity or metabolic syndrome [68]. For their metabolic syndrome model, they used their previously characterized female heterozygous +/− mice lacking endothelial nitric oxide synthase (eNOS), which after feeding with a high-fat diet for 4 weeks develop metabolic-like syndrome phenotype including obesity, glucose intolerance, elevated systolic blood pressure, low high-density lipoprotein, and high insulin. For obesity model, they used wild-type C57BL/6J female mice fed a high-fat diet from weaning for 4 weeks. The pregnant mice were randomized to receive either MYO/DCI (7.2/0.18 mg/mL, resp.) or water as placebo in control group. Pregnant mice with metabolic-like syndrome showed lower serum glucose levels and leptin levels following MYO/DCI treatment as compared to placebo group. In contrast, pregnant mice with obesity alone did not demonstrate improvement in any of the metabolic parameters as compared to placebo group [68]. It was speculated by the study's investigators that MYO/DCI treatment improves glucose tolerance in metabolic-like syndrome pregnant mice but not in the obese mice, possibly involving its specific effects on the nitric oxide pathway. While the above studies suggest that the beneficial effects of inositols may be reduced in obese population, the majority of studies evaluating the effects of inositol treatment on metabolic as well as reproductive function in PCOS women have not specifically addressed the

potential interaction of treatment response with BMI. Future studies are needed to better characterize inositol effects as a function BMI and investigate the potential mechanism/s underlying this differential response.

2.4. Inositol and ART Outcomes in PCOS. Inositol plays an important role in the follicular microenvironment and affects oocyte maturation and embryo development [24]. Elevated concentration of MYO in the follicular fluid appears to exert a positive effect on follicular maturity and is a marker of good quality oocytes in women with or without PCOS [25, 69]. Recent studies have evaluated the role of inositol in ART outcome in women with PCOS. The data from these studies support the notion that inositol has a beneficial effect on ovarian stimulation and ART outcomes in PCOS patients.

Papaleo et al. investigated the effect of MYO supplementation of 2 g twice a day on ART outcomes in sixty patients with PCOS undergoing ovarian stimulation for intracytoplasmic sperm injection (ICSI) cycles. They found significant reduction in the total number of days of stimulation (11.4 ± 0.9 versus 12.4 ± 1.4, $P = 0.01$), significantly lower peak E2 levels at hCG administration (2,232 ± 510 versus 2,713 ± 595 pg/mL, $P = 0.02$), and reduction in degenerated oocytes (1.0 ± 0.9 versus 1.6 ± 1.0, $P = 0.01$) without compromising oocyte yield in the myo-inositol group in comparison to folic acid alone group [11]. However, no differences were found in fertilization rate, embryo quality, or clinical pregnancy rates between the two groups. The authors also suggested that MYO supplementation may decrease the risk of ovarian hyperstimulation syndrome in PCOS patients [11].

Similar findings were reported by Ciotta et al. in a randomized study in which they evaluated the effects of myo-inositol on oocyte and embryo quality in 34 PCOS patients undergoing IVF/ICSI. Patients in this study were divided into two groups: group A was given myo-inositol (2 g) and folic acid (200 μg) 2 times a day for 3 months, while group B received only folic acid (200 μg). Their results showed lower peak E2 levels at hCG administration, less cycle cancellation, higher number of oocytes retrieved, significantly lower number of immature oocytes, and better quality of embryos with higher number of transferred embryos in group A in comparison to group B [41]. Another recent randomized clinical trial by Unfer et al. aimed at comparing the effects of MYO to DCI on the oocyte and embryo quality in euglycemic patients with PCOS undergoing ovarian stimulation for ICSI. Out of eighty-four women with PCOS in their study, forty-three were given MYO 2 g twice a day and forty-one women were given DCI 0.6 g twice a day. The results showed significantly increased number of mature oocytes, good quality embryos, and total pregnancies in MYO-treated group in comparison to DCI treated group [70]. Similar negative effects of DCI were also noted in a study by Isabella and Raffone who investigated the role of DCI in 54 women diagnosed with PCOS undergoing ICSI. After excluding patients with insulin resistance and/or hyperglycemia, they were divided into 5 groups (10–12 patients/group) with a placebo group and 4 other groups receiving 300, 600, 1200, or 2400 mg DCI daily for 8 weeks. They found significantly increased number of immature oocytes in the three groups that received the higher doses of DCI ($P < 0.04$), with significant reduction in grade I embryos ($P = 0.004$) in DCI supplementation group suggesting the negative effect of DCI on oocyte and embryo quality and worsening ovarian response with increasing DCI dosage in PCOS patients [54]. These data are consistent with the DCI paradox hypothesis, which suggests that in PCOS patients there is depletion of MYO due to accelerated epimerization from MYO to DCI and thus further increase in DCI may be accountable for poor folliculogenesis and oocyte response in these patients [26, 71]. Similar paradoxical findings were noted in PCOS patients undergoing IVF and treated with metformin for 4–8 weeks where metformin reduced the number of oocytes retrieved [27]. The authors suggested that the mechanism behind their results may be increased DCI release in response to metformin [27].

Colazingari et al. studied the role of combined MYO and DCI in comparison to DCI alone in PCOS patients undergoing IVF. They included PCOS patients with BMI less than 28 and FSH less than 10 IU/L undergoing IVF-ET and treated them with MYO combined with DCI in a physiological ratio (1.1 g myo-inositol plus 27.6 mg of D-chiro-inositol) or with DCI alone (500 mg) for 12 weeks. They found reduced number of degenerated oocytes (1.04 ± 1.15 versus 1.82 ± 1.55), better fertilization rate (0.75 ± 0 versus 0.58 ± 0.29) ($P < 0.05$), number of transferred embryos (2.22 ± 0.74 versus 1.67 ± 0.85, $P < 0.05$), and improved embryo quality (0.96 ± 0.83 versus 0.7 ± 0.73, $P < 0.05$) in MYO-DCI treated group in comparison to DCI only treated group [28].

In the largest study to date evaluating the effects of MYO supplementation in PCOS women undergoing IVF, Pacchiarotti et al. randomized 526 PCOS patients into three groups: control (folic acid: 400 mcg, $n = 195$), group A (myo-inositol: 4000 mg, folic acid: 400 mcg, and melatonin: 3 mg daily, $n = 165$), and group B (myo-inositol: 4000 mg and folic acid: 400 mcg daily, $n = 166$). All patients received their treatment from first day of the menstrual cycle until 14 days after embryo transfer. Patients in group A required decreased dose of gonadotropins (group A 2058 ± 233 versus group B 3113 ± 345 versus control group 3657 ± 633, $P < 0.001$) and had enhanced quality of oocytes (group A: 48.2% versus group B 35.0% versus control group 38.2%) and embryos (45.7% in group A versus 30.4% in group B and 25.6% in the control group), suggesting the synergistic effect of MYO and melatonin on improving oocyte and embryo quality [72].

In another study by Rago et al. the combined effect of MYO and α-lipoic acid was studied in PCOS patients with normal BMI who had received MYO alone and undergone ICSI previously. They reenrolled 36 PCOS patients who did not achieve pregnancy and 1 patient who had spontaneous abortion and supplemented them with MYO (2 g) and α-lipoic acid (800 mg) per day for 3 months. In MYO and α-lipoic acid group, significant reduction was noted in immature oocytes (0.2 ± 0.4 versus 1.0 ± 1.5; $P < 0.001$), with improvement in mature oocytes (0.87 ± 0.9% versus 0.81 ± 3.9%, $P < 0.05$) and increase in grade 1 embryos (75.7% versus 57.7%; $P < 0.05$) and higher number of pregnancies achieved (52% versus 33.3%; $P < 0.01$) in comparison to MYO alone group [73]. While this study is limited by lack of a control group, the results suggest that α-lipoic acid may enhance the beneficial effects of MYO in PCOS women. Table 1 summarizes the results of the clinical

Table 1

Authors	Study design	Study size	Population characteristics	Type of treatment	Mean age (years)	Mean BMI (kg/m^2)	Main findings
(Papaleo et al., 2009) [11]	Randomized controlled trial	60 women with PCOS Treatment: 30 Placebo: 30	Women with PCOS as defined by oligo/amenorrhea, hyperandrogenism/nemia, and PCO, undergoing ICSI	Treatment: 2 g myo-inositol twice a day plus 400 mg folic acid Placebo: 400 mg folic acid	Treatment: 36.2 Placebo: 35.4	Treatment: 26.7 Placebo: 26.3	Significant reduction in total rFSH units (26 versus 31.7 IU, $P = 0.016$), number of days of stimulation (11.4 versus 12.4, $P = 0.002$), peak E2 level at hCG administration (2,232 versus 2,713 pg/mL, $P = 0.002$), number of germinal vesicles and degenerated oocytes (1.0 ± 0.9 versus 1.6 ± 1.0), and number of cancelled cycles (1 versus 3, $P = 0.003$) in treatment group versus placebo; no significant differences in mature oocytes, fertilization rate, cleavage rate, embryo quality, implantation rate, clinical pregnancy, or miscarriage rate
(Ciotta et al., 2011) [41]	Randomized controlled trial	34 women with PCOS Group A: 17 Group B: 17	Women with PCOS (Rotterdam criteria) undergoing ICSI or IVF	Group A: 2 g of myo-inositol + 200 μg folic acid twice a day × 3 months Group B: 200 μg folic acid twice a day × 3 months	Age < 40 Mean age: not mentioned	Mean BMI: not mentioned	Significantly reduced total rFSH units, peak E2 level at hCG administration, and cancelled cycles (2 versus 5) in group A versus group B. In group A, higher number of mature oocytes, greater number of oocytes retrieved (12 versus 8.5, $P < 0.05$); significantly higher mean number of transferred embryos with greater number of grade I embryos (30 versus 9, $P < 0.01$) in group A. No significant differences in number of fertilized oocytes and total number of biochemical pregnancies between the two groups
(Unfer et al., 2011) [70]	Randomized trial	84 women with PCOS Group A: 43 Group B: 41	Women with PCOS (Rotterdam criteria) undergoing ICSI	Group A: myo-inositol 2 g twice a day for 8 weeks Group B: D-chiro-inositol (DCI) 0.6 g twice a day for 8 weeks	Group A: 35.5 Group B: 36.5	Group A: 24.6 Group B: 25.3	In group A, significant reduction in total rFSH units (1953.6 versus 2360.5 IU, $P < 0.01$), number of stimulation days (11.1 versus 12.7, $P < 0.01$), and peak estradiol levels (2261.2 versus 2740.0 pg/mL; $P < 0.01$); no cycle cancellation in group A versus 4 in group B ($P = 0.05$); no difference between the total number of oocytes retrieved between two groups; in group A, significantly greater number of mature oocytes (8.21 versus 7.08, $P < 0.05$), number of grade 1 embryos (1.64 versus 0.76, $P < 0.01$), number of pregnancies (22 versus 10; $P < 0.05$); no significant differences in clinical pregnancies (15 versus 5, P = NS) and spontaneous abortions (4 versus 3; P = NS) in group A versus group B, respectively
(Isabella and Raffone 2012) [54]	Randomized controlled trial	54 women with PCOS Placebo: 11 Group A: 10 Group B: 11 Group C: 10 Group D: 12	Women with PCOS (Rotterdam criteria) undergoing ICSI	Placebo group Group A: DCI 300 mg daily Group B: DCI 600 mg daily Group C: DCI 1200 mg daily Group D: DCI 2400 mg daily Treatment given for 8 weeks	Placebo: 36.9 ± 1.5 Group A: 36.8 ± 1.6 Group B: 36.9 ± 1.52 Group C: 36.7 ± 1.57 Group D: 37.0 ± 1.25	Placebo: 24.4 ± 2.8 Group A: 25.2 ± 3.5 Group B: 24.7 ± 3.5 Group C: 25.1 ± 3.1 Group D: 25.6 ± 2.9	Significantly increased FSH dose (IU) administered in the two highest DCI dose groups versus placebo (placebo 2239.7 versus group A 2379.1 versus group B 2305.9 versus group C 2368 versus group D 2983.0); number of stimulation days significantly greater in the 3 higher dose DCI groups versus placebo (placebo 11.4 versus group A 12.1 versus group B 12.5 versus group C 12.9 versus group D 13.8); estradiol levels at hCG administration significantly increased in highest dose DCI group versus placebo (placebo group 1429.69 versus group D 1490.24); no significant differences in number of cycles cancelled or total number of oocytes retrieved; significantly lower number of mature (MII) oocytes in group D compared to placebo group ($P < 0.001$); significantly lower embryo quality in DCI supplemented groups versus placebo ($P = 0.004$)

Table 1: Continued.

Authors	Study design	Study size	Population characteristics	Type of treatment	Mean age (years)	Mean BMI (kg/m^2)	Main findings
(Colazingari et al., 2013) [28]	Randomized trial	100 women with PCOS	PCOS (as per Rotterdam criteria) patients with BMI < 28 and FSH < 10 IU/L undergoing IVF-ET	Group A (n = 47): MYO 550 mg and DCI 13.8 mg orally twice a day for 12 weeks Group B (n = 53): DCI 500 mg orally twice a day for 12 weeks	Not mentioned	Not mentioned	Decreased dose of rFSH (1,569.0 versus 1,899.2 IU; $P = 0.04$) and lower E2 levels before hCG administration (2,185.09 versus 2,519.85, $P = 0.05$) in the MYO group versus DCI group, respectively; the number of oocytes retrieved was higher in the DCI group (8.35 in the MYO-DCI group versus 10.75 in the DCI group); reduced number of degenerated oocytes in MYO-DCI group (age < 35: 1.04 versus 1.82; age > 35: 1.00 versus 1.45); fertilization rate was higher in MYO-DCI treated group (0.75 versus 0.58 in the DCI treated group; $P < 0.05$). Greater number of transferred embryos in group A versus B (2.22 versus 1.67, $P < 0.05$); higher embryo quality in MYO-DCI treated group (0.96 versus 0.7, $P < 0.05$)
Pacchiarotti et al., 2016 [72]	Randomized, controlled double-blind trial	Control (n = 195) Group A (n = 165) Group B (n = 166)	PCOS (Rotterdam criteria), FSH < 12 IU/L, and BMI (20 to 26) undergoing first time ICSI	Control (folic acid 400 mcg) Group A (MYO 4000 mg + folic acid 400 mcg + melatonin 3 mg daily) Group B (MYO 4000 mg + folic acid 400 mcg); given from cycle day 1 until 14 days after ET	Control: 32 ± 3.6 Group A: 31.2 ± 2.1 Group B: 31.5 ± 2.8	Control: 22.8 ± 1.3 Group A: 22.8 ± 1.3 Group B: 23.1 ± 1.7	Less total gonadotropin dose (IU) administered in group A 2058 versus group B 3113 and versus control group 3657 ($P < 0.001$); increased number of mature oocytes reaching MII stage in group A (48.2%) versus group B (35.0%) and control group (38.2%); increased percentage of grade I embryos in group A (45.7%) versus 30.4% in group B and only 25.6% in the control group
Rago et al., 2015 [73]	Prospective study	37 women with PCOS	PCOS patients based on 2 of the Rotterdam criteria (PCO morphology and oligomenorrhea) undergoing IVF	MYO (2 g) and α-lipoic acid (800 mg) per day for 3 months in previously MYO-treated group	18–42 years	<24.9	No differences in total dose of FSH administered and duration of stimulation between two groups (1501.9 versus 1498.0 IU; $P = 0.961$, and 11.6 versus 10.8; $P < 0.05$, resp.); in MYO and α-lipoic acid group, significant reduction in immature oocytes (0.2 versus 1.0; $P < 0.001$), with improvement in mature oocytes (0.87% versus 0.81%, $P < 0.05$) and increase in grade 1 embryos (75.7% versus 57.7%, $P < 0.05$) in comparison to MYO alone group; no difference in fertilization and cleavage rates between groups; greater number of pregnancies in MYO and α-lipoic acid group in comparison to MYO alone group (52% versus 33.3%, $P < 0.01$)

ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization; ET, embryo transfer; PCOS, polycystic ovary syndrome; PCO, polycystic ovaries; MYO, myo-inositol; DCI, D-chiro-inositol; BMI, body mass index; rFSH, recombinant follicle stimulating hormone; E2, estradiol; hCG, human chorionic gonadotropin; IU, international units; MII, metaphase II.

trials on the effects of inositols on ART outcomes in PCOS women.

In summary, current data on inositol supplementation and ART outcomes in women with PCOS is limited to small randomized clinical trials which suggest beneficial effects of MYO on folliculogenesis with improved oocyte maturation and embryo quality. However, data on the effects of MYO supplementation on implantation, pregnancy, and live birth rates following ART in these women is scarce. Thus, large clinical trials aimed at assessing these important ART outcomes are needed. In addition, further studies are needed to determine the optimal ratio of administered MYO : DCI resulting in maximal beneficial effect.

3. Conclusions

In conclusion, myo-inositol is an insulin sensitizer which appears to have beneficial effects on ovarian function and response to ART in women with PCOS. It induces nuclear and cytoplasmic oocyte maturation and promotes embryo development. In contrast, D-chiro-inositol appears to exert opposite and detrimental effects on the ovary. While accumulating evidence suggests that myo-inositol improves the number of mature oocytes retrieved, oocyte quality, and embryo quality in women with PCOS undergoing ART, data on its effects on pregnancy and live birth rates in these women is much more limited. Further research on larger patient populations is needed to determine whether inositol supplementation, possibly in combination with other drugs, could improve clinical pregnancy and live birth rates in PCOS women undergoing ART. It is an affordable, widely available, and easy to administer agent which has the potential of improving the outcomes of fertility treatments in women with PCOS.

Competing Interests

The authors declare that they have no competing interests.

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Clinical Study

Myoinositol Improves Embryo Development in PCOS Patients Undergoing ICSI

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The aim of this study was to investigate the activity of myoinositol, in a court of 217 PCOS women undergoing intracytoplasmic sperm injection (ICSI), on pregnancy rate, embryo development, estradiol, and progesterone concentration in blood serum, superoxide dismutase (SOD), and catalase (CAT) in follicular fluid. Concerning the court of patient, 112 (groups I and II) out of 217 were PCOS women, whereas group III consisted of healthy subjects (not PCOS). Group I patients were treated with 400 μg of folic acid per day for 3 months before ICSI, whereas group II patients received 4000 mg of myoinositol and 400 μg of folic acid per day for 3 months before ICSI. Group II revealed a shorter embryo/blastocyst development period between microinjection and 5-cell stage compared to group I. The difference in SOD concentration between groups I and II and between groups II and III was statistically significant. In group II, 34.62% of pregnancies were obtained, whereas in group I this number reached 20% (NS). Myoinositol increased embryo development dynamics and accelerated blastocyst stage reaching time; however, no effect was shown on clinical pregnancy. Furthermore, it restored SOD concentration, lowered in PCOS women, but did not exert any effect on CAT concentration.

1. Introduction

Polycystic ovary syndrome (PCOS) is the most commonly reported ovulation disorder in women [1–3]. It is characterized by considerable heterogeneity in both clinical and hormonal signs [4]. In certain cases, the in vitro fertilization (IVF) is the main treatment for patients with this particular diagnosis.

Success of IVF depends on multiple factors [5] and especially on obtaining a good quality of embryos. Oestradiol and progesterone are two crucial hormones engaged in ovum development. Proper concentrations of these hormones in blood serum ensure a reproductive success in assisted reproductive technology (ART) [6].

An antral follicle constitutes a microenvironment, where ovum develops, which simultaneously influences the future quality of embryo. Oxidative stress plays an important role during folliculogenesis and oogenesis, although our knowledge related to its effects remains still insufficient [7]. It is also well known that PCOS is often associated with certain disorders within the redox system, which depend on the activity of superoxide dismutase (SOD) and catalase (CAT) enzymes, next to other factors [8].

As far as PCOS is concerned, it was possible to confirm several degrees of insulin resistance reported in patients, which could also influence fertility treatment success [1–3, 9, 10]. Myoinositol (myo-Ins), as a mediator of the insulin activity, is considered today a therapeutic agent commonly administered in IVF for the ovulation induction [11–14]. Since myo-Ins is a secondary transmitter of intracellular activity of folliculotropic hormone (FSH), it was assumed that PCOS patients have a deficiency of this hormone, which leads to a disturbance of FSH signalling, causing worsened quality of oocytes, as highlighted in a seminal review, recently published [15]. Therefore, myo-Ins deficiency may also affect the dynamics of embryonic development, as well as the IVF procedure efficiency in patients with PCOS [15].

The primary outcome of this study was to evaluate the influence of myo-Ins on the pregnancy rate and on the dynamics of embryo development in PCOS patients undergoing ICSI. As secondary outcome, our study aimed at testing the myo-Ins effects on oestradiol and progesterone

concentration in blood serum, as well as on SOD and CAT concentration in the follicular fluid of these patients. We considered not only the well-known mechanisms of action, proper to myo-Ins, based on the improvement of insulin and FSH signalling, but also their possible activity as antioxidant molecule.

2. Materials and Methods

This retrospective study was carried out on the data of patients undergoing ART in 2013 and 2014 at the Ovum Fertility Treatment Centre in Lublin. The study covered 217 women treated for infertility by means of intracytoplasmic sperm microinjection (ICSI) technique. All patients were qualified to undergo ICSI due to a moderate, masculine infertility factor that makes it impossible to conduct classic IVF. A total of 198 from treated pairs had 4 to 6 previous intrauterine inseminations during the last 1-2 years that did not lead to pregnancy, and in the case of 19 women we have reported bilaterally blocked fallopian tube. For the subjects enrolled into the PCOS groups, the inclusion criteria required that such patients had to meet the Rotterdam criteria (2004). Women constituting the studied group were between 27 and 35 years old; they all had FSH < 10 IU/mL and appropriate AMH (Anti-Müllerian hormone) value. The exclusion criteria were the following: presence of severe endometriosis, BMI < 17 and >30, and metabolic diseases as well as lowered ovarian reserve.

The study obtained a consent of Bioethical Committee at the Institute of Rural Health in Lublin. All patients signed an informed consent before participating to the study.

All patients were treated with the ICSI procedure, taking advantage of fresh oocytes and fresh sperm.

GnRh analogues (Diphereline: Ipsen Pharma) and FSH recombinants (Gonal-F: Merck-Serono, Puregon: Organon) were used in short protocols to stimulate ovulation from the 3rd cycle day (to a maximum of the 17th cycle day). In the day of implementing ovulation induction, the oestradiol (E_2) (pg/mL) and progesterone (ng/mL) levels were detected in the morning. These two parameters were determined when the largest oocyte in the evaluation of ultrasound exceeded 17 mm diameter. The puncture was conducted 36 hours after administering recombinant HCG (r-hCG) (Ovitrelle: Merc-Serono). A total of 52 patients (group II) among the PCOS subjects were administered with 4000 mg of myo-Ins and 400 μg of folic acid (folate) (Inofolic: Temapharm, Poland) for 3 months before undergoing ICSI. The other PCOS subjects were treated with 400 μg of folic acid alone.

Follicular fluid was collected from follicles with diameter exceeding 17 mm. When the follicular fluid did not contain ovum or was contaminated with blood, the sample was excluded from the study. SOD and CAT were determined on puncture day or on collection day of oocytes, which means between the 11th and the 19th cycle day. SOD activity was measured spectrophotometrically using SOD Assay Kit (Sigma-Aldrich), whereas CATs were detected with the Catalase Assay Kit (Sigma-Aldrich) according to the manufacturer instructions. The final level of the SOD activity and CAT activity was reported as a unit of enzymatic activity per protein mg (mIU/mg).

Oocytes were separated from cells in the granular layer, which was followed by the ICSI procedure 3 hours after ovarian puncture. The inseminated cells were grown in 25 μL drops of Cleavage medium (COOK, Sydney IVF, Australia) under mineral oil in an automatic 5% CO_2 incubator at 37°C until the second day (stage of 2–5 cells). Fifty hours after the ICSI, the culture medium was changed with blastocyst medium (COOK, Sydney IVF, Australia).

Embryo culture was evaluated by means of constant monitoring performed in 10-minute intervals with a camera placed inside the incubator. During all the observation period, the embryos remained in the incubator. The t_0, t_F, and t_C times were defined as the hour of the ICSI, the first moment when pronuclei became visible, and the last moment of their visibility, respectively. The moment when a single cell embryo appeared after syngamy was defined as t_1, and then the superseding divisions as t_2, t_3, t_4, t_5, t_6, t_7, and t_8. The beginning of morula formation was called t_M, whereas t_B is when the first signs of blastocyst cavity could be seen. Blastocysts were evaluated according to criteria declared by ASRM and ESHRE, and only one of them was transferred in order to avoid multiple pregnancy. During the 7th week of pregnancy, the echo of the embryo and heart rate were evaluated by means of ultrasound examination.

3. Statistical Analysis

The measurable parameters were shown as mean and standard deviation, whereas the nonmeasurable ones were presented as numerical amount and percentage.

For qualitative features, a Chi^2 test was utilized to detect any existing differences. The ANOVA variance analysis was used to test differences among groups, whereas R Pearson's correlation was used to verify dependence between embryo development times and selected parameters. The difference was considered statistically significant at $p < 0.05$. The database and statistical tests were conducted using Statistica 9.1 programme (StatSoft, Poland).

4. Results

Patients were divided into the following groups: group with PCOS women ($n = 112$) and group with healthy patients ($n = 105$). In the first group, myo-Ins was administered to 52 women (treated group), whereas 60 were treated with 400 μg of folic acid alone (control group). Both treatments were daily and lasted for 3 months before ICSI. Among the healthy subjects (healthy group), we obtained 33.33% of pregnancies, whereas among the PCOS women this value reached 26.79%. These differences were not statistically significant ($Chi^2 = 1.107$, df = 1, and $p = 0.293$) (Figure 1(a)). However, PCOS patients, under myo-Ins administration, achieved 34.62% pregnancies, whereas in controls just 20% of pregnancies were recorded. These differences were statistically insignificant ($Chi^2 = 3.034$, df = 1, and $p = 0.0810$) (Figure 1(b)).

Table 1: Embryo development times.

	PCOS patients treated with folic acid (group I)	PCOS patients treated with myoinositol plus folic acid (group II)	Patients without PCOS (group III)	p	Difference between groups
t_F	10.38 ± 3.08	9.57 ± 3.24	8.86 ± 2.96	0.010	I-III
t_C	26.14 ± 3.29	22.53 ± 3.98	23.83 ± 3.60	<0.001	I-II, I-III
t_1	26.03 ± 3.30	23.46 ± 3.61	24.23 ± 3.54	<0.001	I-II, I-III
t_2	29.14 ± 3.63	26.60 ± 3.73	25.86 ± 3.25	<0.001	I-II, I-III
t_3	39.43 ± 4.74	35.53 ± 5.83	36.58 ± 4.61	<0.001	I-II, I-III
t_4	42.85 ± 6.69	37.04 ± 4.02	38.45 ± 5.38	<0.001	I-II, I-III
t_5	51.84 ± 7.98	48.16 ± 8.10	53.72 ± 7.28	<0.001	I-II, I-III
t_6	54.03 ± 7.02	53.48 ± 7.41	55.85 ± 8.11	0.130	
t_7	58.05 ± 9.43	57.64 ± 8.51	57.86 ± 8.88	0.972	
t_8	60.50 ± 11.43	63.17 ± 13.36	62.58 ± 12.54	0.468	
t_9	76.82 ± 11.03	74.72 ± 12.22	76.19 ± 11.65	0.619	
t_M	83.79 ± 11.25	85.70 ± 11.23	85.57 ± 11.94	0.584	
t_B	107.56 ± 4.40	104.59 ± 1.66	104.92 ± 2.32	<0.001	I-II, I-III

Differences between embryo development times in groups without PCOS and groups with PCOS, both taking and not taking myoinositol.

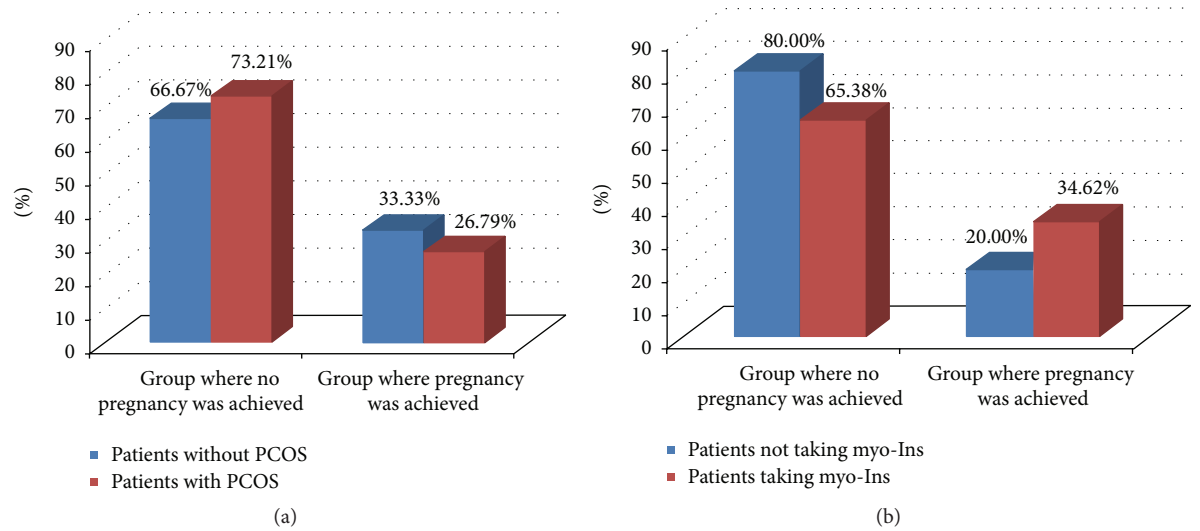

Figure 1: Pregnancy percentage. (a) Percentage layout of pregnancies in the group with PCOS and without PCOS. $Chi^2 = 1.107$, df = 1, and $p = 0.293$; (b) percentage layout of pregnancies in the group with PCOS among patients taking myoinositol and patients who were not taking this preparation ($Chi^2 = 3.034$, df = 1, $p = 0.081$).

When we compared the embryo development times between the groups of PCOS controls (group I), PCOS patients treated with myo-Ins (group II), and healthy subjects (group III), statistically significant differences were observed: t_F ($F = 4.743$, $p = 0.010$) between group I and group III and t_C ($F = 14.724$, $p < 0.001$) between group I and group II, as well as groups I and III. Of note, statistically significant differences were observed between group I and group II, as well as group I and group III, in the following times: t_1 ($F = 8.388$, $p < 0.001$), t_2 ($F = 17.287$, $p < 0.001$), t_3 ($F = 9.762$, $p < 0.001$), t_4 ($F = 18.135$, $p < 0.001$), t_5 ($F = 9.123$, $p < 0.001$), and finally t_B ($F = 19.326$, $p < 0.001$), as shown in Table 1. In the remaining times, no statistically significant differences were observed.

The values of oestradiol in the day of ovulation induction significantly differed between group I and group III ($F = 6.558$, $p = 0.002$), whereas in the same day progesterone did not show significant differences. The concentration of SOD in follicular fluid revealed statistically significant differences among the three groups, namely, between group I and group II as well as between group II and group III ($F = 24.051$, $p < 0.001$). On the contrary, no significant differences were observed for CAT in follicular fluid (Figure 2).

The influence of oestradiol, detected in serum, on the embryo development times showed negative correlations between its level in group I and t_F ($r = -0.307$, $p = 0.017$), t_4 ($r = -0.321$, $p = 0.013$), t_5 ($r = -0.316$, $p = 0.014$), and t_B ($r = -0.312$, $p = 0.015$). In the above-mentioned

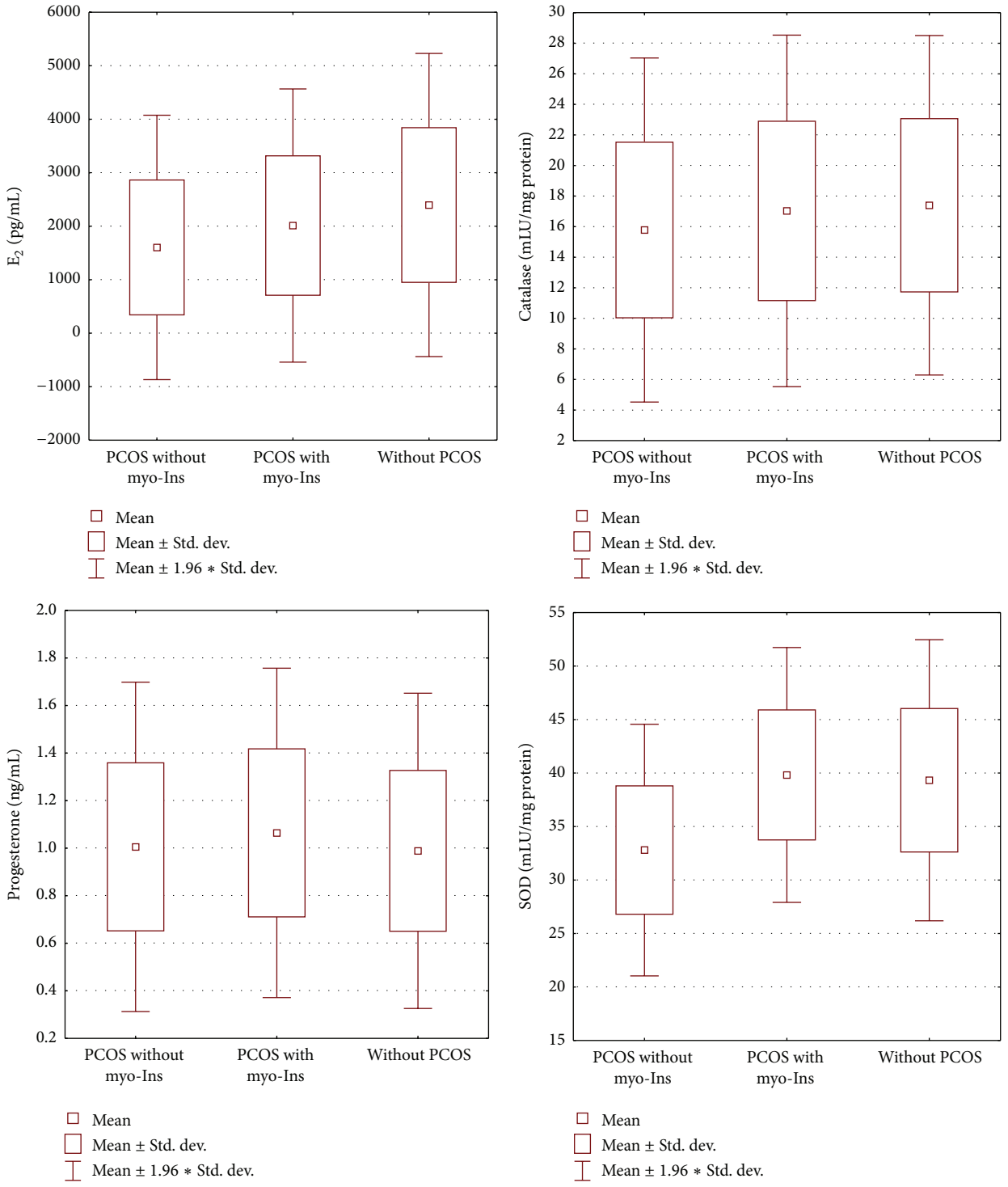

FIGURE 2: Parameters levels. Differences between oestradiol, progesterone, SOD, and CAT levels in groups with PCOS not taking myoinositol (group I) and taking myoinositol (group II) and in group without PCOS (group III).

times, the increase in oestradiol level exerted a considerable influence on embryo developmental dynamics; however, no effect was observed in the remaining times. In group II, similar negative correlations concerned E_2 and t_1 ($r = -0.358$, $p = 0.009$), t_3 ($r = -0.294$, $p = 0.035$), t_4 ($r = -0.283$, $p = 0.042$), and t_5 ($r = -0.365$, $p = 0.008$). In the other times no such statistically significant correlations were detected. Group III showed negative correlations only between E_2 and the following times: t_4 ($r = -0.247$, $p = 0.011$), t_6 ($r = -0.221$, $p = 0.023$), and t_B ($r = -0.192$, $p = 0.049$). In group I a positive correlation between progesterone level and t_2 ($r = 0.284$, $p = 0.028$) was found, whereas in group II negative

correlations between progesterone level and t_F ($z = -0.303$, $p = 0.029$) and t_5 ($z = -0.309$, $p = 0.026$) were reported, and in group III a negative correlation between progesterone level and t_8 ($z = -0.196$, $p = 0.045$) was reported, next to a positive correlation with t_B ($z = 0.253$, $p = 0.009$). No other statistically significant correlations concerning progesterone level appeared. In PCOS patients treated with myo-Ins (group II), researchers observed a negative correlation between SOD levels in follicular fluid and the following times: t_F ($r = -0.318$, $p = 0.013$), t_C ($r = -0.273$, $p = 0.035$), t_1 ($r = -0.484$, $p = 0.000$), t_2 ($r = -0.402$, $p = 0.001$), t_3 ($r = -0.332$, $p = 0.010$), t_4 ($r = -0.400$, $p = 0.002$), t_5 ($r = -0.408$, $p = 0.001$), t_9 ($r = -0.285$, $p = 0.027$), and t_B ($r = -0.341$, $p = 0.008$). In the other times, no significant dependencies were observed. Moreover, in these subjects no significant correlations between SOD and embryo developmental times were discovered. In healthy patients a negative correlation was observed for the same parameter only with t_M ($r = -0.335$, $p = 0.000$). In group I positive correlations were observed between CAT levels and t_5 ($r = 0.338$, $p = 0.008$) as well as t_B ($r = 0.279$, $p = 0.031$), and nothing else. In group II we can see positive correlations between the activity of CAT and t_F ($r = 0.335$, $p = 0.015$) and t_1 ($r = 0.293$, $p = 0.035$), next to t_5 ($r = 0.627$, $p < 0.001$). In group III positive correlations between CAT concentration and t_1 ($r = 0.376$, $p < 0.001$), t_2 ($r = 0.4$, $p < 0.001$), t_3 ($r = 0.197$, $p = 0.044$), and t_4 ($r = 0.274$, $p = 0.005$) were noted, without other significant correlations (Table 2).

5. Discussion

Our study proved that myo-Ins-based therapy, beside its well-known insulin-lowering action, increases the dynamics of embryo development, as well as the activity of SOD in follicular fluid. On the other hand, myo-Ins did not modify CAT activity in follicular fluid. It is important to highlight that SOD activity maintains the balance of redox system, by eliminating superoxide anions and creating H_2O_2. This is the first line of protection against free radicals [8, 16]. The effects on SOD are in perfect agreement with the antioxidant activity which can be ascribed to myo-Ins. Although there is not experimental evidence obtained in mammals, some interesting results coming from researches in fishes have demonstrated that myo-Ins administration can enhance antioxidant defences. In such way, it reduces lipid peroxidation and protein oxidative damage. Various data have shown that myo-Ins opposes detrimental ROS activity and enhances immunity. This molecule looks to activate Nrf2 signalling which plays a key role in inducing gene transcription of antioxidant enzymes and therefore in keeping the physiological redox status [17]. It is a dynamic balance, essential to maintain a healthy condition, and its breaking leads to several pathologies and tissue damage. Among these harmful effects, the abnormalities of redox system stand as the main cause underlying the alterations of genetic material in the ovum [7, 18]. Only the ovum is capable of repairing DNA damage in the spermatozoon; however, this depends on the severity and type of irregularity, as well as the amount of oocytes [19]. It can be therefore expected that myo-Ins treatment may protect against adverse epigenetic changes, and effects of such changes can be evaluated only by examining the condition of a child conceived with the ICSI method [20, 21].

Embryo development times that we were able to obtain during our measurements can be compared with the times that Azzarello et al. and Kirkegaard et al. described in literature [22, 23].

In studies conducted by Gerli et al. the researchers were able to compare a group of 45 women, treated with myo-Ins and folic acid, with 47 women treated only with folic acid. myo-Ins group revealed significantly higher oestradiol levels when compared with the untreated group [13].

Papaleo et al. compared protocols concerning ICSI stimulation with rFSH (recombinant FSH) and Myo-ins with 30 rFSH obtained from 30 patients and reported a lower oestradiol concentration in myo-Ins group, which is not compliant with results obtained by Gerli et al. and the results obtained in our study [12, 13]. They also observed a higher percentage of pregnancies per cycle in myo-Ins group: 33.3% when compared to 13.3% in the group without myo-Ins. In our studies the percentage of pregnancies in the group with myo-Ins was almost identical (34.62%) to the one obtained by Papaleo et al., whereas it reached 20% in the control group.

Chiu et al. conducted studies on the group of 53 female patients taking myo-Ins during stimulation before undergoing IVF and they observed a lowered level of myo-Ins in follicular fluid collected from samples with immature oocytes [11]. Their studies indirectly confirm our results, as far as the influence exerted by myo-Ins on the dynamics of embryo development is concerned.

Seleem et al. compared SOD concentration in follicular fluid of 20 PCOS patients and in a group of 20 healthy patients during the ICSI procedure, and they reported statistically significant higher SOD levels in the group without PCOS, in accordance with our results [24].

In the studies conducted by Muñoz et al., 774 IVF cycles were analysed by means of the technology based on observing embryos in real time, as we made in our study [25]. Authors reported the existence of a significant influence exerted by oestradiol levels on embryo development. Similarly, to our studies, they observed a considerable influence of high E_2 values on increasing the embryo development times; however, they used a different method of statistical analysis and a diverse study model (they divided patients into four groups, depending on the E_2 value in serum, and then they compared the times of embryo development between particular groups) to analyse their results. In this way, it was impossible to unambiguously relate their results. Similarly, to our findings, Muñoz et al. reported that progesterone concentration in blood serum exerts an influence on the dynamics of embryo development only to a slight extent.

Studies devoted to fertility treatment in PCOS patients require further tests, using more numerous groups of patients and with more standardised diagnostic techniques to understand in-depth some specific points; however, the studies so far performed have confirmed the validity of using myo-Ins in ART with PCOS patients.

Table 2: Embryo development times and parameters levels.

		PCOS patients treated with folic acid (group I)				PCOS patients treated with myoinositol plus folic acid (group II)				Patients without PCOS (group III)			
		E_2	Progesterone on the day of ovulation induction	SOD	Catalase	E_2	Progesterone on the day of ovulation induction	SOD	Catalase	E_2	Progesterone on the day of ovulation induction	SOD	Catalase
t_1	r	−0.164	0.038	−0.484	0.082	−0.358	0.128	−0.036	0.293	−0.055	−0.055	−0.001	0.376
	p	0.210	0.775	<0.001	0.533	0.009	0.366	0.801	0.035	0.579	0.581	0.993	<0.001
t_2	r	−0.130	0.284	0.402	0.036	−0.146	0.273	−0.092	0.190	−0.057	0.085	−0.093	0.400
	p	0.323	0.028	0.001	0.787	0.303	0.050	0.515	0.178	0.561	0.390	0.343	<0.001
t_5	r	−0.316	−0.122	−0.408	0.338	−0.365	−0.309	0.154	0.627	−0.084	0.137	−0.016	0.159
	p	0.014	0.352	0.001	0.008	0.008	0.026	0.276	<0.001	0.392	0.163	0.868	0.106
t_M	r	0.028	−0.041	−0.053	−0.014	0.234	0.159	0.120	0.043	−0.136	0.086	−0.335	−0.137
	p	0.831	0.758	0.689	0.915	0.096	0.260	0.396	0.765	0.168	0.382	<0.001	0.162

This table shows only the significant correlations between embryo development times and the level of oestradiol and progesterone in serum and SOD and CAT in follicular fluid.

6. Conclusions

The use of myo-Ins before and during IVF stimulation increases the dynamics of embryo development in the first two days of culture and reduces the amount of time required to achieve the blastocyst stage. However, the influence of these results in the number of clinical pregnancy was not confirmed.

The concentration of superoxide dismutase in follicular fluid is lower in PCOS women when compared with healthy patients; however, when PCOS women are treated with myo-Ins, this concentration reaches values that are observed among healthy women. The effect of oestradiol concentration in serum, as well as the activity of SOD in follicular fluid, increases the dynamics of embryo development. On the other hand, myo-Ins did not have any influence on CAT activity in follicular fluid. SOD increase may be explained by means of the antioxidant activity exerted by myo-Ins, whereas its inefficacy on the rise of CAT levels should be investigated in-depth. The results we obtained confirm also the necessity to conduct further studies focusing on myo-Ins therapy.

Competing Interests

The author declares that he has no competing interests regarding the publication of this paper.

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Clinical Study

Effect of Myoinositol and Antioxidants on Sperm Quality in Men with Metabolic Syndrome

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This prospective longitudinal study investigated the effects of a dietary supplement in patients affected by reduced sperm motility (asthenospermic males) with metabolic syndrome. The product tested was Andrositol®, which contains myoinositol (MI) as principal compound, in association with other molecules, and the parameters evaluated were semen characteristics as well as hormone and metabolic profiles. The inclusion criteria were subjects aged over 18 years, with asthenospermia and metabolic syndrome. The exclusion criteria were presence of cryptorchidism, varicocele, and prostatitis. For this study, 45 males who had such features were enrolled. Their selection was made according to the 2010 World Health Organization (WHO) criteria (5th Edition) for the Evaluation of Human Semen. Hormone and metabolic profiles and semen parameters were assessed at the beginning of the study and after three months of treatment with Andrositol. The differences between the values before and after the supplementation were found statistically significant. Andrositol normalized the metabolic profile of these patients, improving their insulin sensitivity. Moreover, testosterone levels were increased and the semen characteristics, such as sperm concentration, motility, and morphology, highly improved. In conclusion, the association of MI with other molecules (micronutrients and vitamins) could be an effective therapy for metabolic disorders, as well as hormonal and spermatic changes responsible for male infertility.

1. Introduction

Myoinositol (MI) is a sugar-like molecule and it is one of the precursors for the synthesis of phosphatidylinositol polyphosphates (PIPs), key biomolecules belonging to the signal transduction system of several cellular functions [1]. MI exerts a variety of clinical implications, in respect to several pathological conditions, such as metabolic syndrome (MS) and other diseases associated with it [2, 3]. MS is a complex disorder, characterized by alterations in carbohydrate metabolism, obesity, systemic arterial hypertension, and dyslipidemia [4]. These alterations may affect different neuroendocrine axis controlled by hypothalamus and pituitary [4–6].

Recently, a bidirectional interaction between MS and asthenospermia has been proved [7]. In asthenospermia, the proportion of motile sperms is below the World Health Organization's (WHO) standard leading to infertility [8]. High reactive oxygen species (ROS) levels in the semen might be an etiologic factor for male infertility [9]. It is estimated that 25% of infertile men possess high levels of semen ROS. A fertile man does not have high levels of semen ROS instead [10, 11]. ROS are needed for capacitation, acrosome reaction, and ultimately fertilization [12]. However, their uncontrolled production is dangerous for a variety of biomolecules such as lipids, amino acids, carbohydrates, protein, and DNA. High ROS levels negatively affect sperm function [13] due to DNA damage [14, 15]. Furthermore, they reduce sperm motility [16] and impair membrane integrity [17, 18]. Several therapies attempted to counteract the conditions leading to infertility; unfortunately, the totality were found ineffective, except the treatment with some antioxidants [19, 20].

MI supplementation was demonstrated to be really effective in improving semen parameters *in vitro* [21, 22] in oligoteratoasthenozoospermic (OAT) patients.

The present study attempts to determine, for the first time, the effects of a dietary supplement containing MI, selenium, and L-arginine in asthenospermic patients affected by MS. The authors evaluated the effects of this administration on semen parameters, as well as on hormone and metabolic profiles.

2. Patients and Methods

2.1. Patients. This is a prospective longitudinal study of asthenospermic males with MS, under treatment at Altamedica IVF Unit, Rome, Italy. All participants have signed an informed consent form. The inclusion criteria were subjects aged over 18 years, with asthenospermia and MS. The exclusion criteria were presence of cryptorchidism, varicocele, and prostatitis. Overall, 45 males were enrolled from January to April, 2011. Asthenospermia was defined according to the 2010 World Health Organization criteria (5th Edition) for the Evaluation of Human Semen. The following parameters, established by the National Cholesterol Education Program, were used to assess whether the subjects were affected by MS: fasting plasma glucose ≥ 100 mg/dL or diagnosis of diabetes; waist circumference > 102 cm in males; blood pressure ≥ 130/85 mm Hg; triglycerides ≥ 150 mg/dL; HDL cholesterol < 40 mg/dL in males.

The medical histories of all patients were taken into consideration and physical examinations were conducted by the same physicians.

Hormone and metabolic profiles as well as semen parameters were evaluated in the relevant study at the beginning and after three months of therapy. The patients were treated by a dietary supplement administered twice a day containing 1 g MI, 30 mg L-carnitine, L-arginine and vitamin E, 55 μg selenium, and 200 μg folic acid (Andrositol, Lo.Li. Pharma s.r.l., Rome).

2.2. Samples. In order to determinate metabolic and hormonal profiles, the semen and blood samples were obtained from all patients before and at the end of treatment. The semen samples, obtained by masturbation after 3 to 5 days of sexual abstinence, were analysed immediately after complete liquefaction. Each patient was asked to provide three samples, taken up in different days, with the purpose to reduce the variability due to the use of drugs and alcohol or the presence of fever in the days before the test.

Sperm features were evaluated by the same examiner, according to the World Health Organization guidelines (World Health Organization, 2010).

The homeostasis model for insulin resistance (HOMA) index was calculated as the product of the fasting plasma insulin (mIU/mL) and glucose (mmol/L) concentrations divided by 22.5 [23]. Waist circumference (WC), body mass index (BMI), and triglycerides (TG) were determined prior to and after MI supplementation.

Plasma luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol (E2), sex hormone-binding globulin (SHBG), free testosterone, and testosterone (T) levels were measured by radioimmunoassay (RIA). These hormones may contribute to changing sperm concentration, total motility, and morphology [24, 25].

Table 1: Metabolic and hormone profiles before and after treatment.

Analysis	Before	After	*P* value
Metabolic profile			
BMI (kg/cm^2)	28.1 ± 3.5	27.0 ± 3.1	NS
WC (cm)	107.1 ± 4.2	105.3 ± 3.3	NS
Triglycerides (mg/dL)	175.4 ± 12.5	173.2 ± 13.4	NS
HOMA index	2.8 ± 1.2	1.6 ± 0.7	<0.001
SBP	135.3 ± 12.7	128.9 ± 11.0	NS
DBP	91.2 ± 9.2	82.6 ± 9.3	NS
Hormone profile			
LH (mIU/mL)	2.5 ± 1.3	3.3 ± 1.2	<0.01
FSH (mIU/mL)	3.4 ± 1.2	3.5 ± 1.1	NS
E2 (pg/mL)	32.4 ± 5.2	20.9 ± 3.3	<0.01
SHBG (nmol/mL)	55.0 ± 4.9	35.8 ± 3.5	<0.001
Free testosterone (pg/mL)	33.0 ± 11.1	47.2 ± 13.0	<0.001
Total testosterone (ng/mL)	2.8 ± 1.2	3.7 ± 1.4	<0.02

Data are mean ± standard deviation. BMI: body mass index; DBP: Diastolic Blood Pressure; E2: estradiol; FSH: follicle-stimulating hormone; HOMA: homeostasis model for insulin resistance; LH: luteinizing hormone; NS: not statistically significant; SBP: Systolic Blood Pressure; SHBG: sex hormone-binding globulin; WC: waist circumference.

2.3. Statistical Analysis. The results are presented as mean ± standard deviation. The differences in variables before and after MI supplementation were statistically analysed with Student's paired *t*-test. $P < 0.05$ was considered statistically significant. GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) was employed for the data analysis.

3. Results

Table 1 shows patients' metabolic and hormone profiles. Although statistically significant differences were not observed in BMI, waist circumference, or triglyceride levels before and after the treatment with Andrositol for three months, the HOMA index significantly decreased after the therapy ($P < 0.001$).

In regard of hormone profile, only FSH levels were not affected by the supplementation, whilst plasma, E2, and SHBG levels decreased significantly after the treatment ($P < 0.01$ and $P < 0.001$, resp.). Following the treatment, authors observed that a statistically significant increase in LH levels ($P < 0.01$), as well as in free ($P < 0.001$) and total testosterone levels ($P < 0.02$), occurred. Table 2 shows the semen characteristics evaluated before and after Andrositol administration.

After the treatment, all sperm characteristics were significantly improved ($P < 0.001$ for sperm concentration, motility, and morphology).

Table 2: Semen analysis before and after treatment.

Semen parameter	Before	After	*P* value
Concentration (10^6/mL)	16.2 ± 3.4	20 ± 4.2	<0.001
Motility (%)	39.6 ± 6.1	51.4 ± 7.2	<0.001
Normal morphology (%) (normal)	24.9 ± 2.0	30.1 ± 2.3	<0.001

Data are mean ± standard deviation.

4. Discussion

Herein, the authors have determined the effects of a treatment for three months by using a dietary supplement containing MI, in association with other micronutrients and vitamins on metabolic and hormone profiles and on semen parameters of asthenospermic patients with MS.

MS is a combination of disorders, including obesity, that can increase the risk of developing cardiovascular disease and diabetes [26, 27]. It is largely known that components of MS induce reproductive axis disorders [27], though this strong link remains still unclear [28].

Insulin resistance is likely to be responsible, along with adipose tissue mediated signs, for the regulation of gonadal function, causing changes in hormone and proinflammatory cytokines levels [4, 29–31].

Male infertility is frequently due to asthenospermia which ensues a reduction in sperm motility [32].

Furthermore, this condition is generally associated with decreased T, plus elevated E2, FSH, and LH levels [25].

To date, this is the first *in vivo* study that focuses on this topic. Nevertheless, several evidences of MI clinical efficacy in women with PCOS [33–35] and in postmenopausal women with MS are available in literature [12, 14].

The administration of this dietary supplement normalized the metabolic profile of asthenospermic patients with MS, increasing their insulin sensitivity without significant changes in BMI, WC, and triglycerides levels. Moreover, in these subjects, the treatment increased T levels and significantly improved semen characteristics.

It is noteworthy that reduced T levels have been associated with MS and particularly obesity [36]. We may speculate that such decrease is caused by the increased transformation of androgens to estrogens (e.g., E2), by means of aromatization in the peripheral deposits of fat [37, 38]. This imbalance, together with elevated SHBG levels, ends up causing male infertility. It is a consequence of a reduction in sperm concentration and motility, as well as an alteration of sperm morphology [25, 39]. It is interesting to note that the decreased T concentrations constitute in men one of the predicting factors for the onset of insulin resistance, type 2 diabetes, and MS [40, 41].

A replacement therapy with T may improve this condition in these patients; however, it is not completely effective for the treatment of infertility in men [42, 43].

Therefore, new drugs and molecules that increase insulin sensitivity, raising T levels at the same time, are desirable.

Recent studies [44] suggested the involvement of inositols in spermatozoa maturation as well as in their migration from the epididymis. It is interesting to notice that MI concentration is significantly higher in tubules than in serum and other organs [44]. In line with these observations, low MI levels within epididymis and in seminal fluid are associated with reduced fertility [45]. Moreover, MI plays a role in the chemiotaxis and human sperm thermotaxis through the activation of PLC. It results in production of $InsP_3$ and calcium channels opening leading to an increase in Ca^{2+} intracellular concentrations in the flagellum [46]. A recent study highlighted that MI improves OAT samples quality by removing amorphous material. That is likely to be responsible for the high viscosity of seminal fluid [32].

Our clinical study has demonstrated its effectiveness for MS as well as ameliorating the hormonal and spermatic conditions which imply male infertility. As shown, patients with asthenospermia and MS definitely improved their condition after MI use.

The association of selenium and arginine with MI appears particularly interesting. In fact, selenium is a micronutrient important for the male gonad and the process of male reproduction [47, 48]. This element exerts an antioxidant activity mediated by several selenoproteins involved in crucial physiological processes (reproduction, aging, immunity, etc.) [49]. The phospholipid hydroperoxide glutathione peroxidase (PHGPx) plays a pivotal function for male fertility by preserving the cells, undergoing rapid division from oxidative stress as well as stimulating important processes of differentiation [50]. It was demonstrated that PHGPx is necessary for stabilizing the sperm mitochondrial collar and protecting the phospholipids of the germ cell membrane from peroxidation [51].

Also a deficiency in L-arginine content is harmful for male fertility since this amino acid is strongly involved in the process of sperm formation [52]. Reduced levels of L-arginine alter sperm metabolism with the consequence of a decreased motility and spermatogenesis [53]. According to these observations, infertile patients show an increased sperm count and motility when they are treated with L-arginine which does not carry side effects [54]. The relevance of this amino acid for sperm was demonstrated *in vitro* with humans, rabbits, and goats [55–57]. In particular, it prevents the peroxidation of sperm membrane lipids subjected to different stress conditions [58, 59].

Moreover, L-arginine is essential to relieve constricted arteries due to its role in generating a mediator called nitric oxide. It was shown that the administration of this amino acid helps the artery dilatation, increasing blood volume [60].

Overall, these clinical results lead us to deem that the success of the therapy with Andrositol could be mainly due to the association of MI with selenium and L-arginine. It is likely that the antioxidant role of these last two molecules has been important for the improvement of sperm parameters. On the other hand, MI may have helped to balance the hormonal and metabolic parameters, as it acts as second messenger regulating the activities of several hormones such as FSH, TSH, and insulin [61]. In conclusion, this dietary supplement has significantly improved the clinical condition of the asthenospermic patients with MS; therefore, its use should be encouraged.

There are several limitations to the present study, such as lack of controls, limited number of patients, brief treatment period, and unsegmented group of subjects with MS. Therefore, in follow-up, larger prospective randomized case-control studies are needed to elucidate the role and effects exerted by MI, selenium, and L-arginine in asthenospermic patients with different clinical presentations of MS.

Competing Interests

The authors have no conflict of interests.

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Review Article

Putative Key Role of Inositol Messengers in Endothelial Cells in Preeclampsia

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Immunological alterations, endothelial dysfunction, and insulin resistance characterize preeclampsia. Endothelial cells hold the key role in the pathogenesis of this disease. The signaling pathways mediating these biological abnormalities converge on PKB/Akt, an intracellular kinase regulating cell survival, proliferation, and metabolism. Inositol second messengers are involved in metabolic and cell signaling pathways and are highly expressed during preeclampsia. Intracellular action of these molecules is deeply affected by zinc, manganese, and calcium. To evaluate the pathophysiological significance, we present the response of the intracellular pathways of inositol phosphoglycans involved in cellular metabolism and propose a link with the disease.

1. Introduction

Immunological alterations, abnormal placental development, and endothelial cell dysfunction are widely accepted as major determinants for preeclampsia, a severe complication of human pregnancy that may be associated with poor maternal and fetal outcomes [1]. Preeclampsia is clinically defined as new hypertension and proteinuria developed in the second half of pregnancy. Other systemic alterations have been shown to be strictly linked to the pathophysiology of the disease, particularly inflammation, insulin resistance, and dyslipidemia [2, 3], which represent, along with hypertension, the main criteria for the diagnosis of the metabolic syndrome [4]. Several studies suggest that the metabolic syndrome contributes also to abnormal placental development and supports in a vicious cycle inflammation and endothelial dysfunction [5]. Mazar et al. [6] showed that increasing severity of the metabolic syndrome in pregnant women correlates independently with the development of preeclampsia, especially severe early-onset disease that is generally characterized by peculiar placental lesions [7]. Typical features of these alterations are an inadequate/incomplete trophoblast invasion of maternal spiral arteries [7] and acute atherosis [8]. Alterations of trophoblast invasion are mainly linked to failure of the extravillous trophoblast to adequately transform the uterine spiral arteries as a result of altered immunological response at the fetal-maternal interface [9, 10]. Uterine natural killer (uNK) cells seem to play a crucial role in the initial stages of spiral artery remodeling through vascular endothelial growth factor C (VEGF-C) [11]. VEGF-C expression in uNK cells is regulated by phosphoinositide 3-kinase (PI3-K)/Akt signaling pathway [12]. Furthermore, impaired invasion has also been hypothesized to be generated by abnormalities of the histiotrophic nutrition of trophoblast cells at early stages [13]. Acute atherosis is characterized by the presence of foam cells and lipid inclusions that resemble atherosclerotic lesions [8], with the important difference that atherosclerosis develops over decades while the lesions found

in placental vessels in preeclampsia accumulate over a few months. These two aspects support a key role of the metabolic syndrome (inflammation and impaired glucose metabolism) in abnormal placental development.

At present, the correlation between endothelial dysfunction and metabolic syndrome in preeclampsia is still elusive. A growing body of evidence is emerging to show that the inositol second messenger system (myo-inositol and D-*chiro* inositol, two stereoisomers) is involved in metabolic and cell signaling pathways [14]. These molecules are precursors of important signaling molecules, including inositol phosphoglycans (IPG) that are also known as IPG-A and IPG-P, containing myo-inositol (MI) and D-*chiro* inositol (DCI), respectively. These second messengers mediate different actions of insulin: IPG-A stimulates lipogenesis, activates acetyl-CoA carboxylase, inhibits cAMP-dependent protein kinase, and modifies the activities of adenylate cyclase and cAMP-phosphodiesterase; IPG-P was shown to exert specific insulin-mimetic properties on the glycogen metabolism through the activation of pyruvate dehydrogenase phosphatase, glycogen synthase phosphatase, and glycerol-3-phosphate acyltransferase (IPG characteristics are summarized in Varela-Nieto et al., [15]). The two forms modulate insulin action and enhance insulin sensitivity [16]. D-*chiro* inositol phosphoglycans P-type (IPG-P) were shown to be highly increased in preeclampsia [17, 18].

Maternal clinical syndrome in preeclampsia (hypertension and proteinuria) derives from an imbalance of circulating angiogenic factors that results in maternal endothelial dysfunction [19]. We have recently proposed a potential convergence of intracellular pathways between angiogenic factors and inositol messengers on protein kinase B (PKB/Akt) [5]. PKB/Akt is a serine/threonine protein kinase that is activated by a variety of stimuli in endothelial cells, including multiple growth factors (VEGF, IGF-1, and HGF), estrogens, reactive oxygen species, mechanical stimuli, and drugs (i.e., statins). PKB/Akt is a well-known antiapoptotic factor, but it also regulates endothelial cell survival, migration, tube formation, and nitric oxide production [20]. Intracellular action of inositol messengers also depends on the concentrations of some elements like calcium and manganese that may promote or impair signaling transduction. In this article, we summarize the role of these elements and propose an overview of this intracellular system.

2. Regulation of the Pyruvate Dehydrogenase Complex (PDC) by Inositol Phosphoglycans

Reversible phosphorylation of proteins regulates many cell functions and abnormal phosphorylation can be associated with altered signaling leading to a variety of pathogenic states. Pyruvate dehydrogenase complex (PDC) is the key interface between glycolysis and the citric acid cycle and is crucial to the generation of ATP, acetyl-CoA, and NADH by mitochondria (Figure 1). The restoration of ATP following its loss during metabolic activity (or in the heart during ischemia) is of critical importance and subsequent ATP generation is dependent on PDC activity. PDC exists in dynamic equilibrium between dephosphorylated and phosphorylated forms (active and inactive, resp.) and the degree of phosphorylation is controlled by PDH phosphatases (PDP 1,2) and PDH kinases (PDK 1–4). PDK4 is selectively upregulated and PDP2 is downregulated in many tissues in response to starvation, diabetes, and insulin-resistant states [22]. We have previously shown that the putative insulin mediator inositol phosphoglycan P-type (IPG-P, containing D-*chiro* inositol) has a sigmoidal inhibitory action on PDK in addition to its known linear stimulation of PDP [21] (Figure 2). Thus, at critical intracellular levels of IPG-P, this sigmoidal/linear model could potentially amplify switchover from the inactive to active form of PDC, and a "push-pull" system that combined with the hormonal control of IPG-P indicates a powerful regulatory function via inhibition of PDK (Figure 3). The detection of bound zinc to the inositol phosphoglycans is of interest in relation to our reported effects of free zinc ions on PDP and PDH kinases [21] and also in the context of the cardioprotective effects of zinc on vascular ischemia-reperfusion injury [23].

3. Role of IPG Trace Metals in the Regulation of PDC and Channeling of Acetyl-CoA into Oxidative or Lipogenic Routes

Many factors need to be considered in order to understand the relationship between IPG-P and IPG-A types in the regulation of the PDH complex (PDC). These include the differing carbohydrate moieties and structures of the IPG and the binding affinities of their associated trace elements Mn^{++} and Zn^{++}. In view of the number of enzymes and their compartmentation within biosynthetic pathways affected by these trace metals, a number of factors also need to be taken into consideration when extrapolating *in vitro* results to potential *in vivo* intracellular actions. Specifically, it is important to evaluate the free versus the total concentration of the metal ions in view of the presence of metal chelators in the assay system; we have to remember the fact that *in vitro* the PDP is in a soluble form and thus accessible to the effector molecules, either trace metal and/or IPG, in contrast to the *in vivo* situation where the PDK is associated with the mitochondrial membrane; and the *in vitro* system used to study effects on the PDP and PDK components of the PDC is not subject to the complex intracellular network of metal trafficking pathways [24, 25]. These authors emphasized not only the broad range of enzymes requiring metal ions for activity but also their diverse location. Luk et al. [24] stated the following: "the current paradigm is that metal ions are not free agents. Rather, these ions are under careful surveillance by systems designed to detoxify and sequester the metal or to escort the ion to its cognate site in a metalloprotein."

It has been proposed that the effects of IPG-A and IPG-P containing zinc and manganese may be specifically linked to formation of acetyl groups via effects of zinc on the regulation of the PDC, that is, to the generation of acetyl groups via the tricarboxylic acid cycle [26, 27], or towards lipogenic pathways activated by manganese [28–30]. The data presented in Figure 2 suggests that Zn^{++} ions may be more

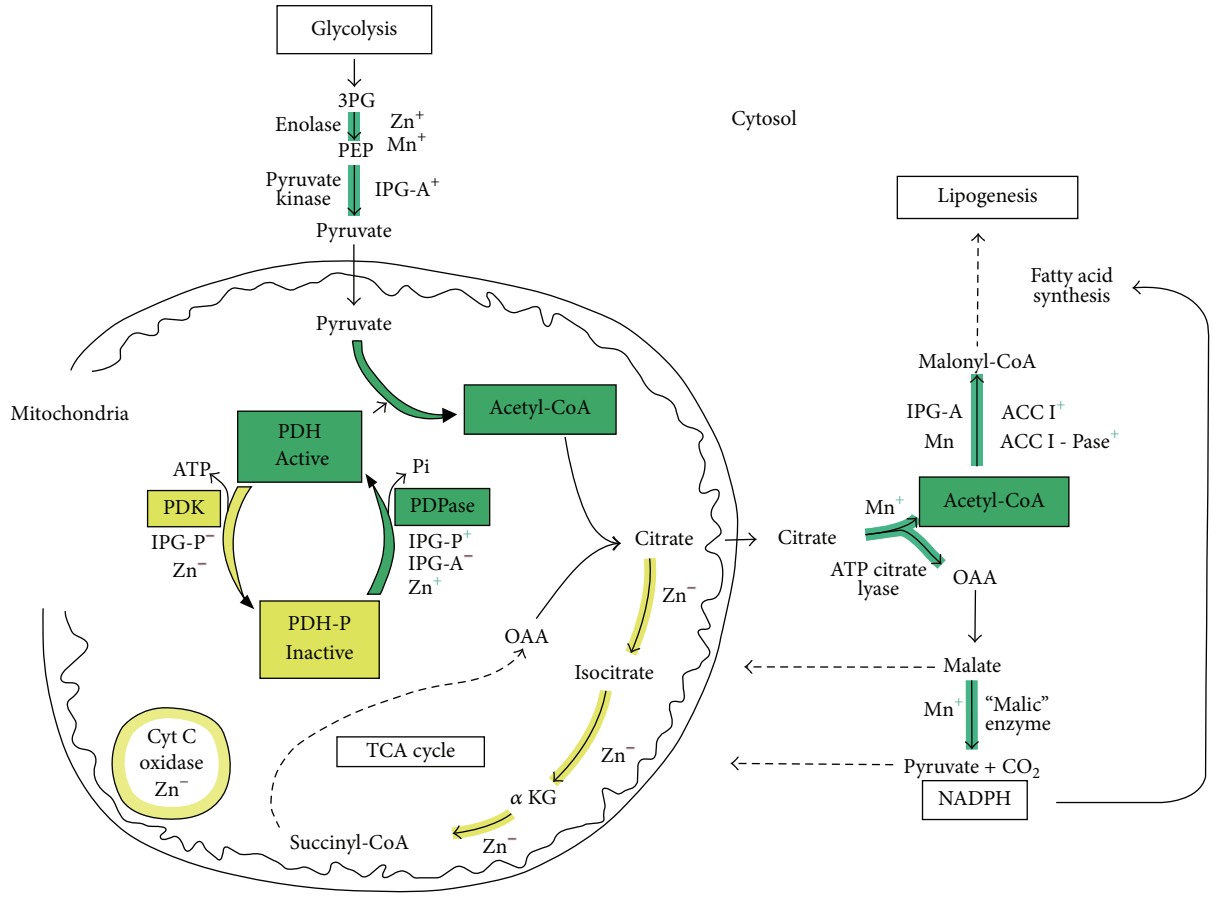

Figure 1: Aerobic glycolysis in the cytosol of cells produces pyruvate that enters the mitochondria where it is oxidized to acetyl-CoA by the pyruvate dehydrogenase complex (PDC). Inositol phosphoglycans (IPG) regulate the activation of PDC and, in its myo-inositol form (IPG-A), promote also lipogenesis in the cytosol. Zinc and manganese hold a key role in this system being involved in most steps as shown.

significant in the regulation of PDC. Maximal activation of PDP is achieved with 0.01 μM Zn^{++} but significant activation is still observed at a concentration of 0.003 μM, a value commensurate with the measured content of zinc in IPG-P isolated from rat liver [21]. Interestingly, this latter value is poised on the steeply rising slope of activation; thus small changes in availability of Zn^{++} could have significant effects on PDP activity. The higher level of Zn^{++} required *in vitro* for the inhibition of PDK may well relate to the association of the enzyme with the mitochondrial membrane *in vivo* and the requirement for a transport system or catalytic chelators to locate the metal ion at the active site. We feel that these differences in inorganic ion concentration do not necessarily detract from the "push-pull" concept of the IPG-P in the control of the PDH complex but rather from limitations of the *in vitro* systems used.

The hyperbolic curve of the effects of Zn^{++} on PDP (Figure 4(b)) falling steeply at concentrations above 0.01 mM may be of significance in relation to the inhibitory effects of IPG-A on the activation of PDP by IPG-P previously reported [31]. IPG-A has a 5-fold higher content of zinc relative to IPG-P and it is suggested that this level of zinc might reach a concentration commensurate with those on the descending arm of the bell shaped curve.

In contrast to the regulation by Zn^{++}, the effect of Mn^{++} on the components of PDC is seen only at considerably higher concentrations (Figure 4(a)). It is notable that IPG-A contains a high content of manganese and this metal is also a known activator of lipogenesis and acetyl-CoA carboxylase [32]. It is suggested that the manganese content of the IPG may be related to the established effects of this trace metal on a range of enzymes linked to lipogenesis. Scorpio and Masoro [33] have shown that the acetyl-CoA carboxylase system is highly sensitive to Mn^{++} and that 50 μM causes almost maximum activation, and approximately 50% maximal activation was shown at 25 μM, a concentration commensurate with the manganese content of IPG-A. In addition, the activation of acetyl-CoA carboxylase by a manganese-dependent phosphatase has been reported [34].

In the present context, the known potent stimulatory effect of free zinc on lipogenesis [35] is considered in relation to the effect of this metal ion on enzymes linked to the formation of acetyl groups and direction of this metabolite to lipid synthesis. We propose that central to this role of zinc is its action as an activator of PDP and inhibitor of PDK thus promoting the conversion of pyruvate to acetyl-CoA. This resetting of the PDC to the active nonphosphorylated state and formation of acetyl-CoA may be enhanced by the effect

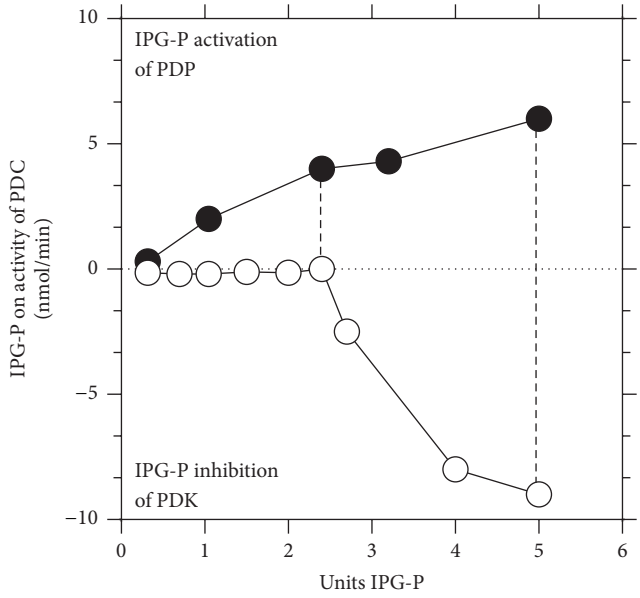

Figure 2: Inositol phosphoglycans P-type (IPG-P), which contains D-*chiro* inositol, can regulate the pyruvate dehydrogenase complex (PDC) by activating pyruvate dehydrogenase phosphatase (PDP) and inhibiting pyruvate dehydrogenase kinase (PDK) in a linear and sigmoid dose-dependent manner, respectively.

of zinc on the activation of enolase in the glycolytic pathway [36] and by the regulation of the phosphorylation state of the insulin receptor [37] and thus downstream pathways favor the provision of pyruvate from glucose. The counter effect of zinc in depressing acetyl-CoA oxidation via inhibition of key enzymes of the tricarboxylic acid cycle including aconitase, NAD-linked isocitrate dehydrogenase, alpha-ketoglutarate dehydrogenase, succinate oxidation, and cytochrome c oxidase [26] could have the effect of both depressing ATP formation and preserving citrate for export to the cytosolic compartment and driving acetyl-CoA towards the lipogenic route. The existence of such metabolic pathways has increased interest in the insulin-mimetic actions of zinc complexes on adipocytes *in vitro* [38] and potential use in the treatment of type 2 diabetes and metabolic syndromes [39].

It is proposed that manganese, present in high concentrations in IPG-A type, could coordinate the conversion of acetyl-CoA to malonyl-CoA and lipid synthesis via manganese activation of ATP-citrate lyase, acetyl-CoA carboxylase 1, and acetyl-CoA carboxylase 1 phosphatase [34, 40]. Manganese is also an activator of "malic" enzyme [41] and thus the provision of NADPH for reductive synthesis and anaplerotic provision of pyruvate. It may be noted that both zinc and manganese are involved in the activation of enolase [36]. Additionally, manganese increases the stimulation by ATP of a putative insulin mediator from liver plasma cell membranes [24] and can largely overcome the regulatory feedback mechanisms of a high fat diet and increase lipogenic enzymes [28]. These observations are in accord with the reported effects of IPG-A on key enzymes of lipogenesis [32].

PDP is divided into two isoforms that respond to insulin and IPG-P, where the isoform 1 requires calcium ions for the activation while the isoform 2 is insensitive to calcium [Roche]. PDP isoform 1 has been correlated with energy production in muscle and heart while PDP isoform 2 acts in starvation and diabetes as demonstrated in the animal model [42]. Measurements of the activity of PDP demonstrate that IPG-P is able to activate the enzyme on both isoforms (Figure 5) and they are optimally activated by the presence of Ca^{++} and Mn^{++} that act in synergy.

4. Discussion

To fully understand the potential role of these messengers in preeclampsia, a comparison with normal pregnancy has to be made. Healthy human pregnancy is characterized by an increasing insulin resistance throughout gestation [43]. Most pregnancies remain well compensated; others develop insulin-resistance related diseases like gestational diabetes and preeclampsia. The role of inositol messengers in insulin resistance is well known in nonpregnant subjects [44, 45] and growing evidence supports a definite role for these molecules in human pregnancy. Placental metabolism holds a pivotal role for the development and maintenance of a healthy pregnancy. Nestler et al. [46] showed that inositol messengers regulate insulin's steroidogenic actions in human placental cytotrophoblasts. In the recent years, inositol messengers were postulated to contribute to carbohydrate metabolism from early stages in human placenta when histiotrophic nutrition takes place [47]. At this time, glycogen and carbohydrate-rich secretions of the endometrial glands represent the main nutrient of trophoblast cells [48]. In fact, during placentation, the trophoblast invades the maternal endometrial glands and submucosal capillary network before reaching the spiral arteries. The first consequence of this is that the oxygen concentration within the intervillous space is relatively low compared to values during the second and third trimester [49] and this prevents a classical aerobic glycolysis (Wharburg metabolism). Inositol messengers were postulated to mediate this situation in the very first weeks of conception, promoting anaerobic metabolism of carbohydrates in human placenta [47]. Mechanisms underlying sugar metabolism through PDC complex have been reported above. Histological studies on healthy placental specimens collected in late first trimester failed to detect DCI in any structure (villi, stroma, and vessels) while a strong staining was found in term placentas [50, 51]. In fact, Kunjara et al. [52] reported a 25-fold increase of DCI concentration in healthy human placenta at term compared to normal liver (standard reference tissue for inositol messengers). It is interesting to highlight that a disrupted intracellular signal related to inositols takes place during preeclampsia. First reports of a relationship between inositol messengers and pregnancy complications were reported by Rademacher's group [52]. They reported with a threefold increase of DCI in placental specimens of preeclamptic mothers compared to healthy samples while no myo-inositol messenger was detected. Histological studies demonstrated a more intensive staining in villous stroma of preeclamptic placental specimens compared to gestation-matched controls [53]. Furthermore, a tendency towards a higher staining in samples of severe early-onset

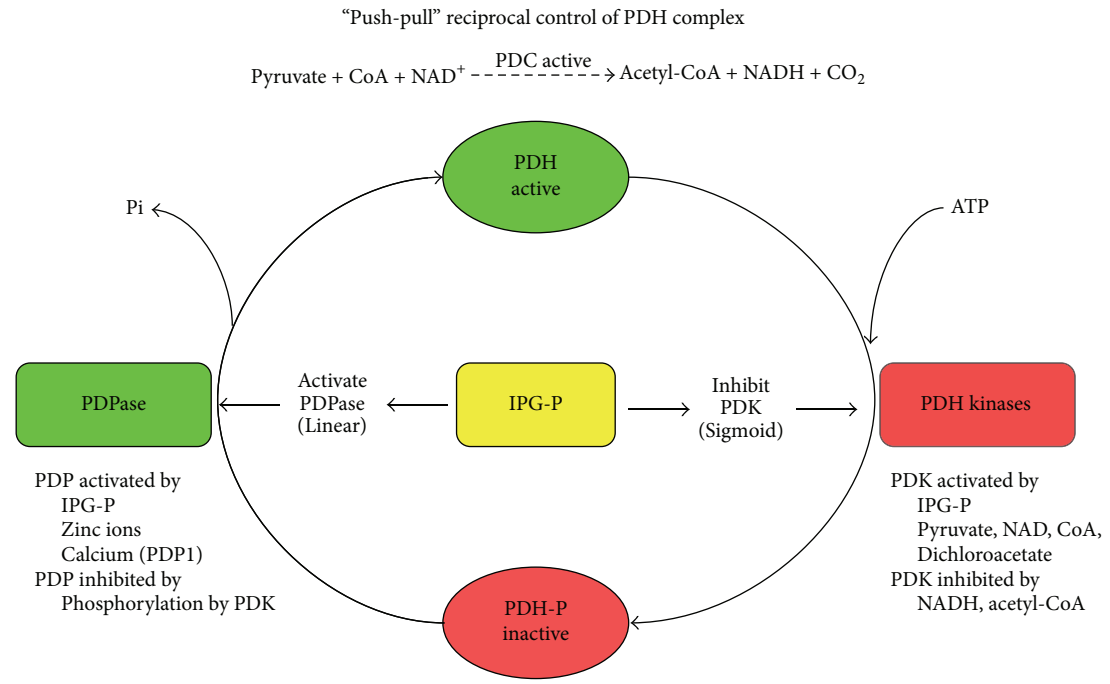

Figure 3: The reciprocal control of the pyruvate dehydrogenase complex has been shown in a previous article of ours [21] and is here represented in a scheme.

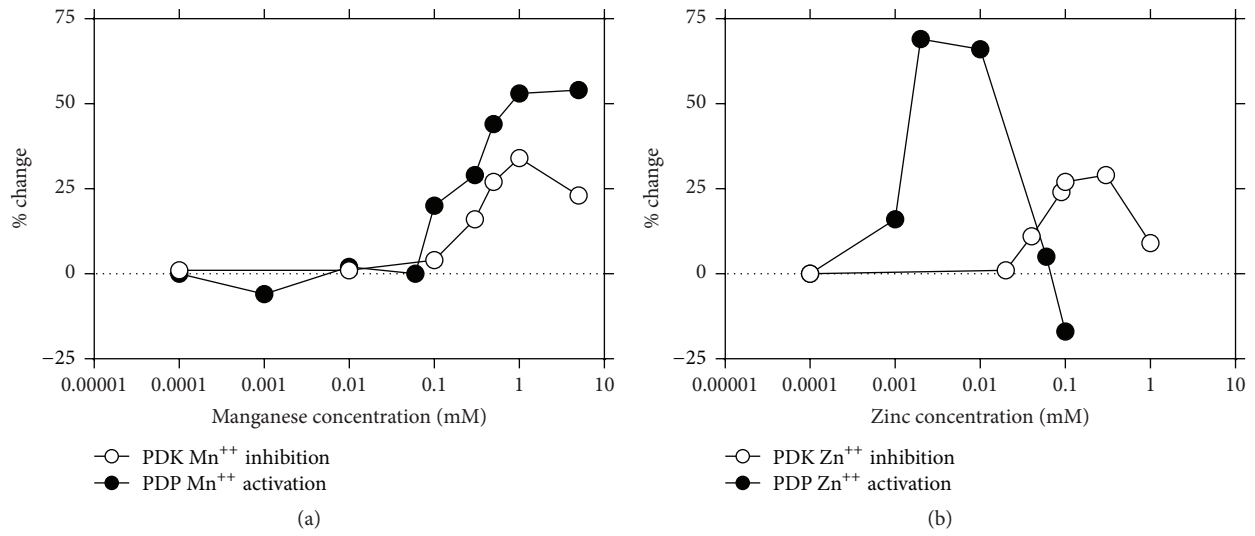

Figure 4: The activation of pyruvate dehydrogenase phosphatase (PDP) and the inhibition of pyruvate dehydrogenase kinase (PDK) are influenced by ion concentrations.

preeclampsia was reported. In fact, it has been demonstrated that insulin treatment of isolated membranes of insulin-sensitive tissues like normal human placental membranes and BC_3H-1 myocytes results in the production of soluble DCI messengers [54, 55]. We confirmed a high release of DCI messengers after insulin incubation of fresh placental membranes from healthy pregnancy but found that there was no release of messengers after insulin stimulus in preeclamptic samples [56]. This may be explained by IRS-1 and IRS-2 inhibition due to serine-phosphorylation and subsequent impairment of downstream insulin signaling in preeclampsia [56]. This altered pathway prevents or impairs glycogen synthase (GS) that is normally activated via PI3K–PDK–Akt–GS kinase-3. Activated Akt also leads to membrane fusion of GLUT4 vesicles and promotes the action of mammalian target of rapamycin (mTOR) kinase that regulates many cell processes such as growth, proliferation, survival, protein synthesis, and gene transcription. Furthermore, DCI can be transported into mitochondria to activate PDH phosphatase, which in turn activates PDH.

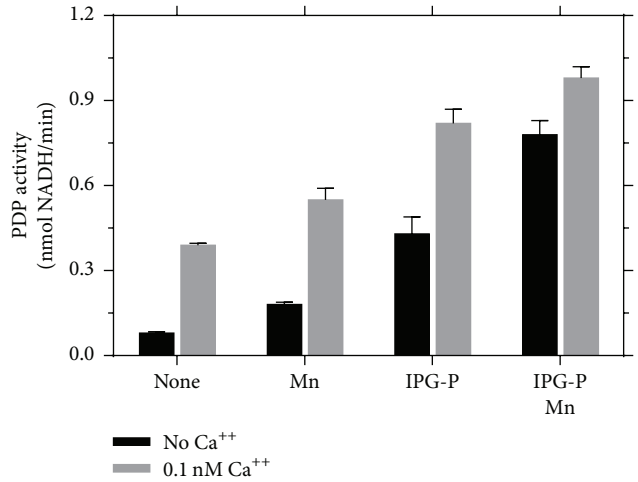

Figure 5: Pyruvate dehydrogenase phosphatase (PDP) can be activated in presence of calcium. This effect is boosted by inositol phosphoglycans P-type (IPG-P) and manganese.

Increased levels of DCI-phosphoglycan (IPG-P) were reported in many maternal and fetal fluids and tissues during preeclampsia (as reviewed by Scioscia et al., [57]). The longitudinal assessment of maternal urine specimens revealed significant increased excretion of DCI-phosphoglycans a few weeks before the onset of clinical preeclampsia [18, 58] and during active preeclampsia [59]. Inactivation of proteins downstream to insulin receptor can be induced by fatty acids, cellular stress, or diverse inflammatory cytokines, including TNFα, IL-1, or lipopolysaccharide [60]. According to our findings, the increased production of DCI-phosphoglycans during preeclampsia may be a compensatory effect to overcome IRS inactivation. Furthermore, hypertension in insulin-resistant states, including preeclampsia, is determined by inadequate vasodilation and paradoxical vasoconstriction through collateral signaling pathways (VEGF, IGF, and insulin) [60–64]. In a recent report, we have shown that insulin and VEGF signaling pathways related to vascular vasodilator/vasoconstrictor effects converge on PKB/Akt [5]. In fact, inhibition of the VEGF/Akt/eNOS pathway blocks VEGF-driven nitric oxide release and promotes vasoconstriction [65]. The activation of the insulin signaling in vascular cells through IRS-1/PI-3K/Akt results in eNOS phosphorylation on Ser1177, leading to enhanced nitric oxide production [66]. Along with this hypothesis, insulin-dependent activation of eNOS through the hypoxia inducible factor 1 was shown to be linked to subsequent increased expression of VEGF-A [67].

PDP activity is strictly related to ion concentrations in mitochondria. The presence of Ca^{++}, Mn^{++}, and Zn^{++} strengthens the response to IPG-P. This may be of importance when increased workload and adrenergic stimulation occurs, for instance, in muscle and heart cells under stress during hyperglycemia and starvation.

Certainly, the interaction between intracellular pathways related to metabolism and vascular alterations is not fully explained yet. We argue that a large part of the pathophysiology of preeclampsia may be based on this interaction given the observation that both aspects have been described in preeclampsia and long-term alterations (higher risk of metabolic syndrome and cardiovascular disease) can certainly represent the final expression of these disrupted cross talk pathways. Whether inositol second messengers are among the key molecules in this process as it seems nowadays has been shown in detail. Many points have been already demonstrated while minor linking aspects should be investigated.

Characterization of the intracellular action of IPG-P in the context of a metabolic disease like preeclampsia helps in understanding the underlying biochemical mechanisms that occur in placenta and endothelial cells. This may help in the characterization of the link between alterations reported in human placenta and the endothelial reaction that occurs in this disease. Indeed, these aspects have to be fully elucidated in human tissues of women with preeclampsia, so further studies are warranted. On the other hand, this may lead to pharmacological interventions to support and/or prevent anomalies that lead to the development of preeclampsia.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Insulin-Sensitizers, Polycystic Ovary Syndrome and Gynaecological Cancer Risk

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Preclinical, early phase clinical trials and epidemiological evidence support the potential role of insulin-sensitizers in cancer prevention and treatment. Insulin-sensitizers improve the metabolic and hormonal profile in PCOS patients and may also act as anticancer agents, especially in cancers associated with hyperinsulinemia and oestrogen dependent cancers. Several lines of evidence support the protection against cancer exerted by dietary inositol, in particular inositol hexaphosphate. Metformin, thiazolidinediones, and myoinositol postreceptor signaling may exhibit direct inhibitory effects on cancer cell growth. AMPK, the main molecular target of metformin, is emerging as a target for cancer prevention and treatment. PCOS may be correlated to an increased risk for developing ovarian and endometrial cancer (up to threefold). Several studies have demonstrated an increase in mortality rate from ovarian cancer among overweight/obese PCOS women compared with normal weight women. Long-term use of metformin has been associated with lower rates of ovarian cancer. Considering the evidence supporting a higher risk of gynaecological cancer in PCOS women, we discuss the potential use of insulin-sensitizers as a potential tool for chemoprevention, hypothesizing a possible rationale through which insulin-sensitizers may inhibit tumourigenesis.

1. Introduction to Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in women of reproductive age, with an estimated incidence rate of 5–10% [1, 2]. The syndrome has a heterogeneous presentation, which includes hirsutism, often related to hypersecretion of ovarian androgens, anovulation, menstrual irregularity, infertility, and pregnancy complications. PCOS may predispose women to cardiovascular and metabolic dysfunction as well as an increased risk of type 2 diabetes [3]. The excess of ovarian androgen secretion [4] may affect raised pituitary luteinizing hormone (LH) production and in addition contributes to the mechanisms of anovulation. Insulin sensitivity and secretion can be affected by hyperandrogenism; however dietary factors and independent genetic seem to have also a role [5]. Hyperinsulinemia and peripheral insulin resistance are present in about half of PCOS women mainly in adipose tissue and skeletal muscle while ovarian theca and granulosa cells have been reported to be highly sensitive to insulin. Insulin stimulates ovarian theca cells to produce androgen (i.e., testosterone) through the stimulation of the insulin receptor (IR) like LH [6]. Both hypersecretion of LH and hyperinsulinemia cooperate to increase ovarian theca cell androgen production contributing to androgen dependent hirsutism also by suppression of hepatic secretion of sex hormone binding globulin (SHBG), which increases the bioavailability of circulating testosterone [7]. The use of antihyperglycemic drugs enhancing peripheral insulin sensitivity is widely used to treat metabolic aspects in PCOS women often from a long time [8]. However, the correction of hyperinsulinemia leads to a decreased ovarian

androgen production. Chan indicates that using insulin-sensitizers may have a role as a tool for cancer prevention [9]. In the present review we try to hypothesize a possible rationale through which insulin-sensitizers may inhibit tumourigenesis (Figure 1).

2. Insulin Receptor Signaling and Phosphoinositide Pathways

Insulin receptor is a transmembrane receptor encoded by a single gene, belonging to the large class of tyrosine kinase receptors [3]. It is activated by insulin, insulin growth factor 1 (IGF-I), and insulin growth factor 2 (IGF-II) [16]. The main activity of the IR when bound by an insulin molecule is inducing glucose uptake. For this reason a decreased sensitivity in insulin receptor signaling, associated with impaired glycogen synthesis and inhibition of glycogen breakdown, progressively leads to metabolic disorders and type 2 diabetes mellitus [17]. Peripheral insulin resistance is then defined by a decrease in insulin-dependent glucose transport at the level of target tissues [18] due to defects at both the insulin receptor and/or postreceptor signaling [19]. Following hormone binding the IR undergoes conformational changes which allow autophosphorylation of its tyrosine residues, docking sites for insulin receptor substrates (IRSs) involved in phosphatidylinositol-3-kinase (PI3K) activation and recruitment to the plasma membrane of the serine/threonine protein kinase Akt/PKB which represents the main intracellular interconnecting pathway activated to ensure insulin biological action together with the mitogen-activated protein kinase (MAPK)/extracellular-signaling regulated protein kinase 1/2 (ERK 1/2) pathway [20, 21]. In mammals, almost five isoforms of the regulatory subunit of PI3K interact with IRSs activating the catalytic subunit and phosphorylating the phosphatidylinositol 4,5-biphosphate [PI(4,5)P2] which in turn enables other phosphoinositide dependent kinases [mainly phosphatidylinositol 3,4,5-trisphosphate, PI(3,4,5)P3] involved in the phosphoinositide signaling system and in the signaling of the glucose transporter type 4 (GLUT4) the main GLUT-like protein identified in mammals [22]. The phosphoinositide signaling system plays a pivotal role in the regulation of cellular processes, such as vesicle transport cell proliferation and cytoskeletal remodeling [23] and its deregulation may lead to many diseases including cancer [24]. Glucose uptake for cellular function is additionally regulated by a sensor of intracellular energy level, the 5′ adenosine monophosphate-activated protein kinase (AMPK) pathway. AMPK is a highly conserved serine/threonine protein kinase that appears to universally exist as heterotrimeric complex comprised of catalytic α-subunit and regulatory β- and γ-subunits [25]. Like several other protein kinases, AMPK and its orthologues are only significantly active when phosphorylated by upstream kinases at the level of a conserved threonine (Thr172) within the kinase domain [26]. In mammals, the main upstream kinase phosphorylating Thr172 is the liver kinase 1-Ste20-Related Adaptor-Mouse protein 25 (LKB1-STRAD-MO25) heterotrimeric complex, a biologically active unit containing the tumour suppressor kinase LKB1 [27, 28]. This complex is constitutively active and presents a high basal activity [29]; however its ability to phosphorylate Thr172 is improved by conformational changes in its AMPK substrate, due to the binding of 5′ adenosine monophosphate (AMP) active to its γ-subunit. Moreover, kinases that can activate AMPK also include calcium/calmodulin-dependent protein kinase (CaMKK) [30] and transforming growth factor β-activated kinase 1 (TAK1) [31]. Overall, AMPK maintains cellular energy homeostasis and its activation by metabolic stress (accountable of energy depletion) leads to inhibition of cell growth and promotion of adenosine-5′-triphosphate (ATP) production. Indeed, once activated, AMPK can encourage catabolic pathways and inhibit anabolic pathways to restore energy stores [32]. Furthermore, many of the biosynthetic pathways, such as synthesis of triglycerides, fatty acids, phospholipids, sterols, glycogen, proteins, and ribosomal RNA, are switched off by AMPK. These pathways are subject to a AMPK dependent downregulation via multiple mechanisms, involving both phosphorylation of pathway key enzymes and longer-term effects on gene expression [32]. As a molecular target of metformin, AMPK is a known key point in the treatment of metabolic syndrome and type 2 diabetes. Recently, AMPK is emerging as a possible metabolic tumour suppressor and a target for cancer prevention and treatment. Moreover, it has been observed that AMPK downstream targets can influence many effector proteins involved in various regulatory processes that contribute to the pathogenesis of cancer. Even if AMPK negatively regulates cyclooxygenase 2 (COX-2), a proinflammatory enzyme associated with tumourigenesis, and can induce phosphorylation of tumour suppressor p53, resulting in cell cycle arrest, the main molecular mechanism involved in the AMPK-mediated tumour suppression is the negative regulation of PI3K/Akt/mTOR signaling pathway [33]. mTOR is a serine/threonine protein kinase that regulates cellular processes including proliferation, growth, motility, survival, protein synthesis, and transcription [34, 35]. mTOR forms two functionally distinct complexes, mTORC1 and mTOR2. mTORC1, a central signaling node that integrates signals arising from growth factors, nutrient availability, and energy status [36], promotes cell growth by phosphorylating ribosomal protein S6 kinase 1 (S6K1) and Eukaryotic Translation Initiation Factor 4E-Binding Protein 1 (4E-BP1) [37, 38]. Inhibition of mTORC1 signaling by AMPK occurs via dual pathways. First, AMPK phosphorylates tuberous sclerosis complex-2 (TSC2) [39] which converts the Ras homolog enriched in the brain (RHEB), a GTP-binding protein, activating upstream mTORC1, to its inactive GDP-bound form. In a second time, AMPK phosphorylates a regulatory-associated protein of mTOR (RPTOR or Raptor), a subunit of the mTORC1 complex, inhibiting it [40, 41]. As AMPK is considered the most important molecular effector of metformin, it may function as an important key-regulator of cellular energy homeostasis. Therefore, it is activated in response to a shortage of energy in order to boost cellular catabolic activities [42]. On the contrary, insulin, which is an anabolic hormone released by nutrient intake, induces cell growth and energy storage. It is therefore not surprising that there is an antagonism between these two pathways. AMPK negatively modulates mTOR through the phosphorylation of TSC2 and Raptor.

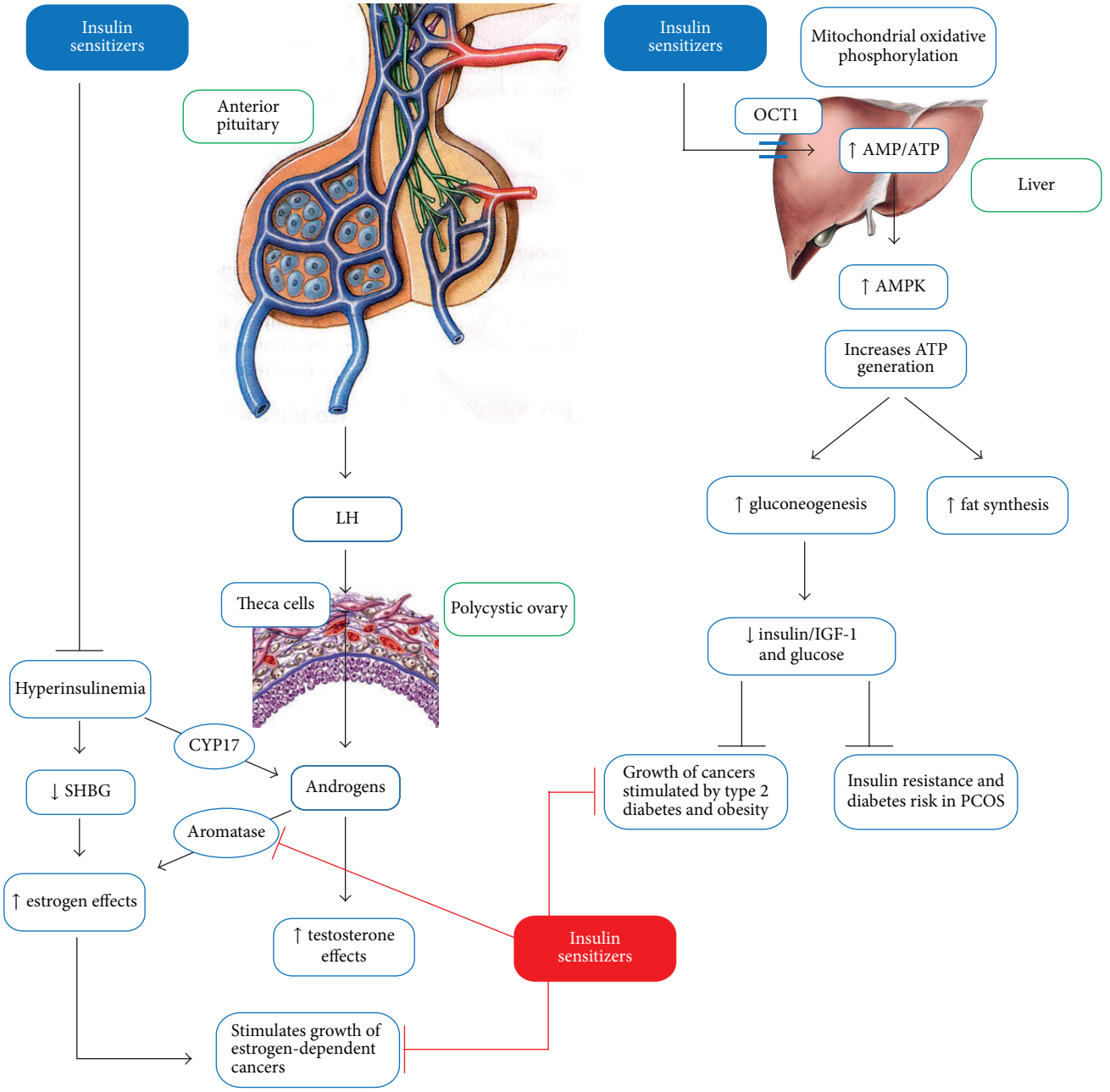

Figure 1: The combined action of insulin-sensitizers on the liver and ovary and the supposed protecting effect on endocrine-related gynaecological cancer. In the liver metformin inhibits mitochondrial respiratory complex 1 promoting AMPK activation which improves the metabolic profile reducing hyperglycemia, hyperinsulinemia, and insulin resistance. Insulin-sensitizers have a positive effect in PCOS patients through normalization of hyperinsulinaemia that otherwise amplifies the excessive androgen production from the ovary theca cells via CYP17 phosphorylation and lower levels of SHBG. Metformin and inositols may play an anticancer role both as insulin-sensitizers and as aromatase inhibitors. Indeed, they improve metabolic profile, inhibiting the growth of tumoural cells stimulated by hyperinsulinemia, and normalize estrogen production, inhibiting the growth of estrogen-dependent cancers. OCT1: Organic Cation Transporter 1; LH: luteinizing hormone; ISF-1: insulin sensitivity factor; CYP17: Cytochrome P-45017; SHBG: sex hormone binding globulin; IGF-1: insulin-like growth factor; PCOS: polycystic ovary syndrome.

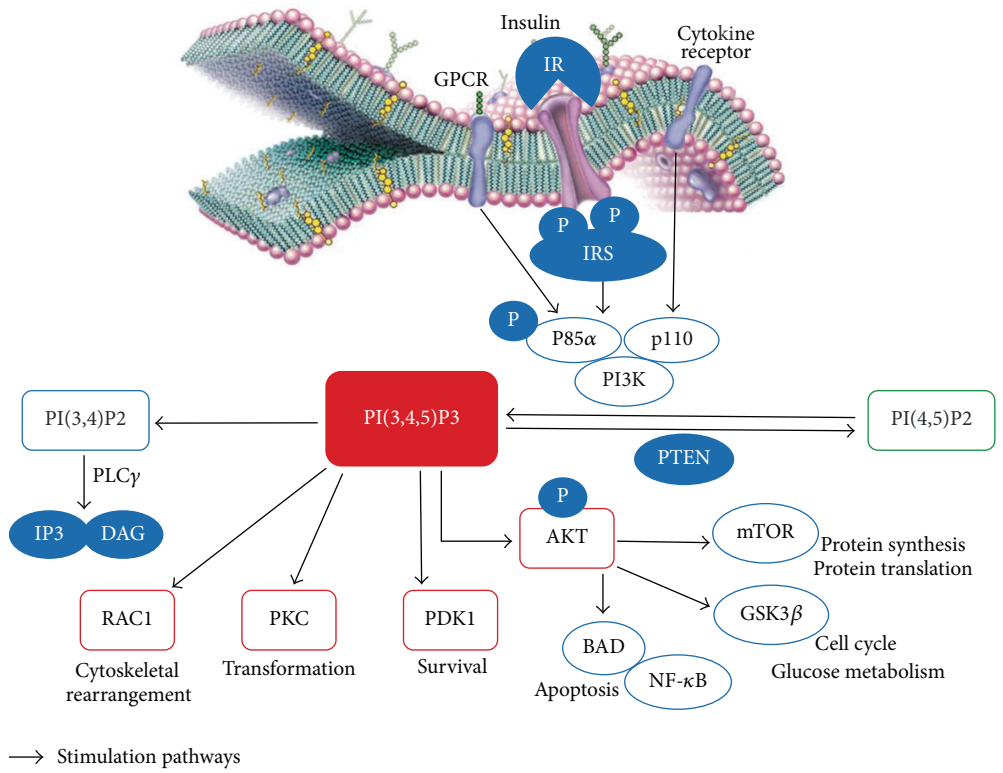

Figure 2: Insulin receptor and phosphatidylinositol (3,4,5)-trisphosphate formation. The activation of PI3K by insulin, G-protein coupled receptor, or growth factors generates different 3′ phosphorylated inositol products which in turn recruit effector molecules regulating many cellular functions involved in cells survival and metabolism. GPCR: G-protein coupled receptor; IR: insulin receptor; IRS: insulin receptor substrate; PI3K: phosphatidylinositol 3-kinase; PI(4,5)P2: phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P3: phosphatidylinositol 3,4,5-triphosphate; PI(3,4)P2: phosphatidylinositol 3,4-bisphosphate; PLCγ: Phospholipase C γ; IP3: inositol trisphosphate; DAG: diacylglycerol; PKC: protein kinase C; PTEN: phosphatase and tensin homolog; Akt: protein kinase B; RAC1: Ras-Related C3 Botulinum Toxin Substrate 1; PDK1: phosphoinositide dependent protein kinase-1; mTOR: mammalian target of rapamycin; GSK3β: glycogen synthase kinase 3; NF-κB: nuclear factor κB; BAD: Bcl2-Antagonist of cell Death.

A similar but functionally opposite dual approach is used by the insulin-signaling pathway to activate mTORC1 and thus contrast AMPK effects [36]. It has been demonstrated that the insulin-activated kinase Akt phosphorylates TSC2 at distinct sites from those specific for AMPK, blocking its RHEB-GAP function [36, 43, 44], and also phosphorylates proline-rich Akt substrate of 40 kDa (PRAS40), enhancing its inhibitory effect on the mTORC1 complex [45]. Hence, AMPK and Akt reciprocally control mTOR pathway. As demonstrated in rat model, the insulin-activated kinase Akt has been shown to phosphorylate the α1 catalytic subunit of AMPK at Ser485, reducing the rate of subsequent Thr172 phosphorylation and the LKB1 induced activation in cell-free assay, whereas the pretreatment with insulin may blunt Thr172 phosphorylation during ischemia in perfused rat hearts [46]. The signaling relationship between Akt and AMPK is quite complex. Akt positively regulates mTORC1 and negatively regulates AMPK while some conditions activating AMPK may silence Akt signaling suggesting a bidirectional cross-talk between AMPK and Akt, even if the functional consequence in terms of tumour progression is unclear [33]. For instance, this bidirectional feedback mechanism was shown by Choudhury et al. using a number of prostate cancer cell lines [47]. Therefore, activated AMPK may inhibit or promote Akt signaling depending on the cellular microenvironment and the following phenotypic consequences may dependent on the tumour and cellular context [33] (Figure 2).

Translating into clinical practice the current research data discussed above, it is possible to support the evidence that metformin might induce growth-static effect on several cancers, including pancreatic cancer [48], glioma [49], prostate, and colon cancer [50]. The metformin antiproliferative effect may be exerted through the inhibition of the PI3K/Akt/mTOR signaling transduction pathway [35, 51–54]. Finally, AMPK can also regulate p53 [55] and modulates the activity of transcription factors and coregulators that control the cell cycle [56, 57]. Current evidence suggests that AMPK can act as a tumour suppressor by modulating inflammations, contrasting the metabolic changes that occur during tumourigenesis and directly inducing cell cycle arrest [58].

3. Insulin-Sensitizers in PCOS and Cancer Prevention

PCOS is associated with insulin resistance [59] and with a certain number of metabolic disorders [60]. Insulin

resistance is a dysmetabolic condition in which a greater amount of insulin is required to exert a physiological cellular response. It is characterized by increased secretion of insulin from pancreatic β-cells and compensatory hyperinsulinemia. The treatment of insulin resistance and hyperinsulinemia includes the use of insulin-sensitizers (metformin, thiazolidinediones, and inositols) [8].

3.1. Metformin. Metformin is a synthetically derived biguanide, off-label used in the management of dysmetabolic disorders and insulin resistance in PCOS. It has been demonstrated that metformin, when used in addition to changes in the life-style, may restore ovulation in women with PCOS and reduces the risk of ovarian hyperstimulation syndrome [8]. The effects of metformin on glucose metabolism seem to be secondary to its actions on the mitochondrial respiratory chain, inhibiting the mitochondrial respiratory complex I [61, 62]. Therefore, it leads to a reduction in ATP production and oxidative phosphorylation, resulting in a higher AMP/ATP ratio which in turn inhibits gluconeogenesis and modulates AMPK. AMPK activation by metformin induces fatty acid oxidation, reduces lipid synthesis, and inhibits gluconeogenesis [63, 64]. AMPK is regulated by metformin via an upstream kinase, LKB1, which is produced by a tumour suppressor gene and controls cell growth. Although AMP-activated protein kinase is one of the most important molecular targets of metformin, this drug also has AMPK-independent effects on glucose metabolism, as it has been shown in mice that deficiency of LKB1 and AMPK following treatment with metformin leads to a reduction of serum glucose levels. These effects might be the result of the change in AMP/ATP ratio that regulates hepatic glucose output upstream of AMPK and suppresses cyclic-AMP-protein kinase A signaling [8]. Although the primary action of metformin is the metabolic homeostasis, its putative role in treating different types of cancer is under investigation [65, 66]. Currently, metformin effect in preventing a number of cancers has been demonstrated by many epidemiological and clinical data [37, 67, 68] but the molecular mechanisms are yet to be elucidated. The treatment of endometrial cancer cells with metformin leads to displacement of constitutively active K-Ras from the cell membrane, uncoupling the mitogen-activated protein kinase (MAPK) signaling pathway [69]. Additional anticancer activity could be its antiaromatase activity [38] which leads to a reduction in circulating estrogen levels in obese women and an upregulation of progesterone receptor expression by endometrial cancer cells [70]. Furthermore, metformin has antiangiogenic effects, directly scavenging free radicals and blocking endogenous reactive oxygen species [71]. A study *in vitro* has shown that it significantly reduces DNA damage and mutation rates [72]; this might be the explanation of the reduced risk of cancer that has been shown by many epidemiological studies through the use of metformin. The potential preventive and therapeutic role of this drug on breast cancer has also been studied. For instance, Jiralerspong et al. in a retrospective study of 2529 diabetic patients, including 68 on metformin and 87 not, showed that diabetic patients affected with breast cancer and treated with metformin had higher rates of complete response to neoadjuvant chemotherapy than those not taking metformin [73]. In cell culture, the proliferation of a wide range of cancer cells such as breast cancer cells is inhibited by metformin [74, 75] and Liu et al. reported the apoptotic effect of metformin in triple negative breast cancer cells [76]. Recently it has been shown that metformin may also target cancer-initiating cells. In particular, it was shown that its use in a subpopulation of breast cancer cells suppressed their growth and decreased the ability of these cells to form tumours in mice [77]; its combination with trastuzumab leads to a reduction of cancer-initiating cell population in Her2-amplified breast cancer cells [78]. Moreover, metformin can regulate breast cancer-initiating cell ontogeny through repression of the process of epithelial to mesenchymal transition (EMT) [79]. Overall, metformin benefits to breast cancer may be due to its role on cellular cycle and PI3K/Akt/mTOR signaling pathway and negative insulin effects on tumour development and growth.

3.2. Thiazolidinediones. Thiazolidinediones (TZDs) used in the treatment of metabolic diseases are a class of drugs also known as glitazones, including troglitazone, rosiglitazone, and pioglitazone [80]. Troglitazone was withdrawn from the worldwide market in 2000 due to an increased incidence of drug-induced hepatitis [81]. Glitazones are synthetic ligands containing a functional group in which thiazolidine serves as a dione and acts as an agonist of the peroxisome proliferator-activated receptor gamma (PPARγ) whose endogenous ligands are free fatty acids (FFA) and eicosanoids [82]. Activated PPARγ shapes a heterodimeric complex with retinoid X receptor (RXR) and binds to peroxisome proliferator hormone response elements, upregulating specific target genes and downregulating others [83]. Thiazolidinediones act as insulin-sensitizing agents, increasing fatty acid uptake and storage in adipose tissue, promoting adiponectin expression, and decreasing expression of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD) which converts cortisone to active cortisol [84]. PPARγ can regulate the transcription and/or the activity of different key regulators of energy homeostasis where it can be considered as a control element of the cellular energy status [82]. Beneficial effects of these drugs are also studied in polycystic ovary syndrome showing that TZDs can reduce insulin resistance in PCOS women mainly acting on the adipocytes and the muscle cells [85, 86]. In addition to improving insulin resistance and compensatory hyperinsulinemia, administration of TZDs may increase the ovulation rate and pregnancy in patients affected with PCOS [87]. It has been observed that TZDs can modulate secretion of several endocrine hormones reducing androgen production or improving gonadotropins secretion [81]. Moreover, Hu et al. showed a weak PPARγ mRNA expression in granulosa cells of PCOS patients, that varied in relation to the clinical features of the disease and was upregulated following administration of different dosages of insulin and/or rosiglitazone [88]. *In vitro* experiments run on human ovarian cells demonstrate the negative regulation exerted by TZDs on steroidogenic enzymes 3-β-hydroxysteroid-dehydrogenase (3β-HSD) and aromatase [80, 89, 90].

Interestingly, as with metformin, glitazones can activate AMPK indirectly and regardless of PPARγ involvement [91],

through an increase of intracellular ADP : ATP ratio, which leads to the inhibition of respiratory chain and subsequent AMPK phosphorylation [62].

Considering the potential effects of glitazones on gynaecological tumorigenesis, Shah et al. showed that thiazolidinediones decrease vascular endothelial growth factor (VEGF) production by human luteinized granulosa cells *in vitro* [92]. On the contrary, another study, which characterized the response of ovarian xenograft tumours to the nonhypercalcemic vitamin-D2 derived anticancer agent (MT19c), observed a reduced efficacy of MT19c and cisplatin following stimulation of PPARγ with rosiglitazone, suggesting that PPARγ promotes survival for some ovarian tumour cells [93]. Overall, these lines of evidence—although limited and contrasting in some aspects—indicate that in some conditions these drugs may be considered as protective agents even if, due to their side effects, their use should come as a secondary option in the treatment of the metabolic problems linked to PCOS.

3.3. Inositols. Inositol (cyclohexane-1,2,3,4,5,6) is a polyol of cyclohexane with six hydroxyl groups, identified for the first time in animal muscle tissue by J. J. Scherer in 1850. In the past it has been considered as a member of the vitamin B complex but inositol cannot be considered a "true" essential nutrient, as it can be synthesized by the human body. Indeed, cells can activate inositol biosynthesis, starting from glucose, through two enzymatic reactions. The first step consists of the conversion of D-glucose 6-phosphate to L-inositol-1P, catalyzed by 1L-myoinositol-1-phosphate synthase (MIPS). Subsequently L-Ins(1)P-phosphatase hydrolyzes L-inositol-1P forming myoinositol (myo-Ins) and orthophosphoric acid [94]. Inositols exist under nine stereoisomeric forms depending on the spatial orientation of their six hydroxyl groups. Myo-Ins and D-chiro-inositol (D-chiro-Ins) are the two main inositol stereoisomers naturally present in animal and plant cells, either in their free form or as bound-components of phospholipids or inositol phosphate derivatives. Myo-Ins is abundantly present in many plant sources and in certain high-fiber diets, such as cereals and legumes. When ingested, it is actively absorbed at the level of the gastrointestinal tract involving an Na^+/K^+-ATPasi [95]. Inositol transporters are involved in uptake and intracellular distribution of inositols that, according to their transport mechanism, can be classified as sodium ion coupled and proton coupled inositol transporters [96]. Myo-Ins is present in the cell embedded in the phospholipids docked to the plasma membrane and it is even a component of the glycosylphosphoinositides layered on the inner surface of the cellular membrane acting as important component of the calcium trafficking [97, 98]. The discovery of the second-messenger function of phosphatidylinositol, generated by the action of cytidine diphosphate diacylglycerol (CDP-DAG) inositol phosphatidyltransferase and its phosphorylated derivatives, the phosphoinositides, marked a turning point in studies of hormone function [99]. Myo-Ins plays an important role in the insulin signal transduction, lipid metabolism, calcium ions flow regulation, and assembly of cytoskeletal proteins. Recently, a substantial body of research evidenced its role in PCOS as an insulin-sensitizing agent affecting different pathways at both ovarian and nonovarian level [100]. In addition to being found in some food, myo-Ins is formed in the cells, its biosynthesis deriving from the conversion of D-glucose-6 phosphatase to L-inositol-1-phosphate [101]. Myo-Ins is an important component of the structural lipids and its various phosphates, including the phosphatidylinositol phosphate (PIP) lipids [102–104], are essential in maintaining the cellular membrane bilayer. Moreover, myo-Ins is the structural basis for many secondary messengers, including inositol triphosphates (InsP3), phosphatidylinositol (PI), polyphosphoinositides [i.e., PI(4)P, PI(4,5)P2, and PI(3,4,5)P3], and inositol phosphoglycans (IPGs), which controls several physiological events including regulation of hormone activities [105]. Inositol pyrophosphates act as a controlling factor of PI3K/Akt signaling pathway [106]. Myo-Ins is involved in mTORC1 and AMPK signaling activities [99]. Currently, myo-Ins is involved in inositol polyphosphate multikinase (IPMK) activity, a key enzyme for inositol polyphosphate biosynthesis and metformin-induced AMPK activation [107] (Figure 3).

In mammals, IPMK is also known as a physiologically important phosphatidylinositol 3 kinase (PI3K) that forms PI(3,4,5)P3 which activates Akt signaling pathway [108]. Recently, Kim et al. suggested the role of IPMK as a novel cofactor for mTORC1 signaling [109]. Moreover, IPMK also appears to be a novel AMPK-binding protein whose binding affinity for AMPK is dynamically controlled by glucose levels [107]. Myo-Ins intracellular homeostasis is adjusted by some processes, mainly the extracellular uptake via specialized myo-Ins transporters, phosphoinositide cycle, de novo biosynthesis from glucose-6-phosphate by 1-D-myoinositol-phosphate synthase (MIPS), and inositol monophosphatase (IMPase), efflux, and degradation [110]. Abnormalities in one or several of these processes lead to myo-Ins intracellular depletion found in conditions of hyperglycemia and insulin resistance as observed in diabetes mellitus [99]. Inositol transporters were identified in bacteria, protozoa, fungi, plants, and animals. According to their transport mechanisms, they can be classified into two groups: sodium-dependent myo-inositol transporters 1 and 2 (SMIT1/2) and proton coupled inositol transporters (HMIT) [105]. Many lines of evidence support the benefits of myo-Ins supplementation for some metabolic disorders associated with insulin resistance, because of its insulin mimetic properties. As mentioned above, PCOS is characterized by metabolic features such as central obesity and insulin resistance with compensatory hyperinsulinemia that also are key factors in the pathogenesis of chronic anovulation [111–113]. Myo-Ins, through its insulin-sensitizing effect, plays an important role in improving metabolic and hormonal parameters in women affected with PCOS [114, 115]. However, the best therapy for this disorder is constituted by the treatment with myo-Ins plus D-chiro-Ins combined in agreement with the physiological myo-Ins/D-chiro-Ins plasma ratio (40 : 1) [116–119], which allows obtaining very interesting results and also avoids some detrimental effects due to D-chiro-Ins alone at high concentrations [120]. Myo-Ins involvement in the reproductive axis function is highlighted by the pivotal role played by inositol (1,4,5)-triphosphate [Ins(1,4,5)P3] in

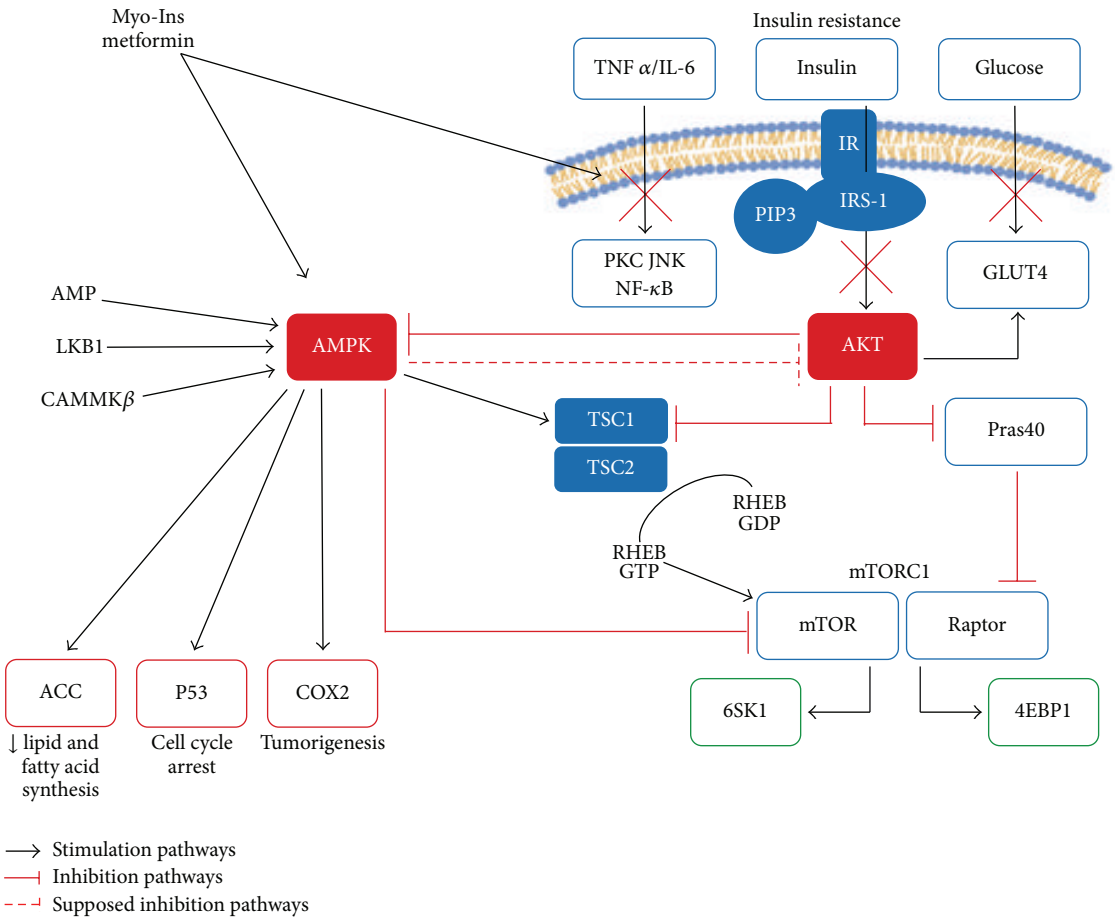

Figure 3: Negative regulation exerted by insulin-sensitizing on the molecular mechanism feeding insulin resistance. Role of AMPK as a metformin and myo-Ins molecular target. AMPK activation induces COX2 inhibition, p53 expression, and mTORC1 silencing through TSC2 and Raptor phosphorylation. The regulating activity of AMPK in processes of tumour induction and progression suggest that metformin may be proposed as an anticancer agent. TNFα: Tumour Necrosis Factor α; IL-6: interleukin 6; IR: insulin receptor; PKC: protein kinase C; JNK: c-Jun N-terminal Kinase; NFκB: nuclear factor κB; IRS-1: insulin receptor substrate 1; PI(3,4,5)P3: phosphatidylinositol 3,4,5-trisphosphate; Akt: protein kinase B; GLUT4: glucose transporter type 4; TSC1: tuberous sclerosis 1; TSC2 tuberous sclerosis 2; AMPK 5′: adenosine monophosphate-activated protein kinase; AMP: adenosine monophosphate; LKB1: liver kinase B1; CaMKKβ: calcium/calmodulin-dependent protein kinase β; Pras40: Proline-Rich Akt/PKB Substrate 40 kDa; RHEB: Ras Homolog Enriched in Brain; mTOR: mammalian target of rapamycin; Raptor: regulatory-associated protein of mTOR; mTORC1: mammalian target of rapamycin complex 1; 6SK1: 6S Kinase 1; 4EBP1: Eukaryotic Translation Initiation Factor 4E-Binding Protein 1; ACC: Acetyl-CoA Carboxylase; p53: Tumour Protein 53; COX2: cyclooxygenase 2.

the regulation of calcium ion (Ca^{2+}) release during oocytes development and its role in meiotic competence and the final stage of oocyte maturation [121]. In PCOS patients, a defect in tissue availability or an altered metabolism of inositol or IPGs mediators may contribute to insulin resistance [122, 123]. As myo-Ins involvement in PI3K/Akt/mTOR and AMPK signaling pathway [124] plays important roles in the regulation of cellular growth and survival it is interesting to examine its possible role in cancer development. Han et al. have shown that regression of bronchial dysplastic lesions can be caused by myo-Ins through inhibition of active Akt and ERK and its molecular target, *in vivo* and *in vitro* [125]. Furthermore, a phase 1 clinical study carried out in order to determine the potential chemopreventive effect of myo-Ins in smokers with bronchial dysplasia showed a potential positive effect at the oral daily dose of 18 gr for three months [126] without significant adverse events despite the high doses used. Indeed, myo-Ins may work through multiple mechanisms to inhibit tumour progression. Interest is growing for inositol hexaphosphate (InsP6), a natural occurring polyphosphorylated carbohydrate contained in high-fiber diets and present in mammalian cells, and for its role in cancer prevention as well as control of experimental tumour growth, progression, and metastasis [127]. Dong et al. reported that InsP6 strongly inhibited cellular transformation induced by epidermal growth factor (EGF), ERK, and PI3K activation [128]. Based on the synergic activity of myo-Ins and metformin in metabolic disorders characterized by insulin resistance, and according to the suggested antitumoural effect of metformin treatment, the possibility that myo-Ins

might have some ability to inhibit tumour growth also in gynaecological cancer should be considered. More studies are needed to clarify a possible anticancer role of myo-Ins in patients affected with PCOS.

4. Insulin-Sensitizers in PCOS and Ovarian Cancer

Ovarian cancer (OC) is the eighth most common cancer and the seventh most common cause of death from cancer in women worldwide. There are factors that increase the risk to develop OC but they are still not well validated. The onset of OC can be caused by many factors such as age, family history of OC, infertility treatment and assisted fertilization, hormonal substitution in menopause, and obesity [129]. Endocrine disorder associated with hypersecretion of ovarian androgens, anovulation, and menstrual irregularity in PCOS seem to be the cause of higher risk of the epithelial ovarian cancer development due to the abnormal hormonal environment. In fact, in PCOS, we see hormonal alteration with abnormal concentrations of unopposed oestrogens. Several hypotheses are made to explain ovarian carcinogenesis. The majority of malignant ovarian tumours seem to have steroid hormone receptors (62% for oestrogen, 49% progesterone, and 69% for androgen) [130]. Berchuck et al. proposed that an interference in the local concentration of growth factors and steroids conducts malignant changes in the ovarian epithelium [131]. Oestrogens prevent apoptosis through Bcl-2 upregulation and this may lead to ovarian carcinogenesis, whereas progesterone has a proapoptotic effect on ovarian epithelium through either the modulation of transforming growth factor β (TGF β) isoform expression or the activation of the Fas/Fas Ligand signaling pathway [132]. Also androgens seem to be involved in ovarian carcinogenesis in animal models and testosterone may intensify the ovarian epithelial tumours growth [133]. Androgens decrease TGF β receptor levels, allowing tumour cells to escape TGFβ1 mediated growth inhibition, thus promoting ovarian cancer progression [134]. Repeated anovulatory cycles, with the generation of inclusion cysts, may increase the risk of genetic damage, because of exposure of the epithelial cells to high concentrations of oestrogens in follicular fluid [135]. Likewise, high levels of circulating gonadotropins increase the risk of developing ovarian cancer, especially in early postmenopausal years [136]. In the literature, few and conflicting studies addressing the possibility of an association between ovarian cancer and PCOS are reported [137], with the additional problem raising from uncertainties in PCOS diagnostic criteria, as most of such studies have been conducted before the Rotterdam ESHRE/ASRM PCOS Consensus 2003 [138]. In 1996, Schildkraut et al. demonstrated in a population-based case-control study a 2.5-fold increase in the risk of ovarian cancer among women with PCOS [139]. A stratified analysis adjusted for age found that oral contraceptive use plays a protective role in women with PCOS [139]. Stratified age-adjusted analysis also showed that younger women reporting to have PCOS were at much greater risk for developing ovarian cancer [139]. However, the small number of women with PCOS ($n = 31$) limited the interpretation of these findings, with the possibility of recall bias in subjects affected with ovarian cancer [140]. An Australia-wide population-based case-control study of subjects aged 18–79 years with new diagnosis of invasive epithelial ovarian cancer ($n = 315$) versus controls ($n = 1508$) tested the hypothesis that hyperandrogenism is associated with the genesis of ovarian cancer. The authors found no evidence that self-reported histories of PCOS were associated with an increased ovarian cancer risk, although women with PCOS who were also overweight had a significantly increased risk of serious borderline tumours [141]. A case-control analysis of 1611 patients versus 9170 controls with a diagnosis of ovarian cancer shows that PCOS (OR 1.63 95% CI 0.65–4.08) was associated with a tendency towards an increased risk of ovarian cancer. A retrospective case-control analysis has shown that long-term use of metformin was associated with lower rates of ovarian cancer (OR 0.61, 95% CI 0.3–1.25) [142]. Identification of a number of proteins overexpressed in both PCOS and ovarian cancer, such as superoxide dismutase, calreticulin, vimentin, fibrinogen γ, lamin B2, and malate dehydrogenase, assured the identification of women with PCOS with an increased risk of developing this malignancy [143]. A systematic review and meta-analysis of observational studies considered for PCOS association and ovarian cancer only three studies [144]. The OR resulted significantly higher in the single study of women aged <54 years [139]; however the increased risk of ovarian cancer in women with PCOS was not significant [144]. This is in line with a nationwide population-based retrospective cohort study, conducted in Taiwan, where also the increased risk of ovarian cancer in the PCOS group was not reported [145]. The analysis of a large cohort study from the Danish National Patient Register compared the women's cancer incidence with that of the general Danish female population by means of standardized incidence ratios (SIRs) and found no association between PCOS and ovarian cancer [146]. The association with obesity is not pronounced as much as the association with endometrial cancer; however a recent meta-analysis and a systematic review found that ovarian cancer was barely more common in women with a BMI $\geq 30\,kg/m^2$ [147, 148] and a prospective cohort study showed increased mortality from ovarian cancer among overweight and obese women compared with normal weight women [149]. Lee at al. reported a higher risk of ovarian cancer in diabetic women, which persisted after adjusting for BMI, age, alcohol intake, and smoking [148]. Numerous prospective observational studies suggested that not only patients with type 2 diabetes taking metformin were at lower risk of developing cancer [150] but also mortality was less common [151]. Stadtmauer et al. suggested that insulin-sensitizing agents use, as well as improving reproductive function, produces long-term health benefits [152]. Also myo-Ins acts as a controlling factor of PI3K/Akt signaling pathway [99] and it is also involved in mTORC1 and AMPK signaling [107]. Thus, besides its insulin-sensitizing effect, it plays an important role in improving metabolic and hormonal parameters in women affected with PCOS and a possible chemopreventive effect can be hypothesized. The identification of a high risk group is important, and this may include women with PCOS,

impaired glucose tolerance, morbid obesity, endometrial hyperplasia, and risk for developing ovarian cancer.

5. Insulin-Sensitizers in PCOS and Endometrial Cancer

A growing scientific interest towards addressing the risk of endometrial cancer in women affected by polycystic ovarian syndrome is emerging in endocrinology and gynaecologic oncology. Currently, endometrial cancer (EC) is the most common gynaecological malignancy in Europe and North America, with a global tendency to further increase over time, mainly involving postmenopausal women [153]. The main risk factors regarding hormone-related cancer are recognized in exposure to unopposed estrogen therapy, overweight and obesity, late-age menopause, and nulliparity [114, 115] and therapy with Tamoxifen as well [154]. Since EC risk is strongly associated with high circulating oestrogen levels, all of the pathological conditions leading to this hormonal status may promote endometrial proliferation. This is the case of women affected by PCOS that show a high risk of developing atypical endometrial hyperplasia (EH), which is considered a precancerous lesion, which may progress to EC. An assessment of the prevalence of EH in PCOS women still remains a widely debated issue. Although the hyperplastic rate has been reported as high as over 48% [155], others [156] did not confirm it, referring to an overall prevalence close to 1%. As already mentioned [157], such a wide range of EH prevalence in PCOS patients may reflect the heterogeneous nature of the PCOS phenotypes and the varying diagnostic criteria over time. The biological relationship between PCOS and EC, although still unclear, refers to a combination of complex reproductive and metabolic disorders: likewise, chronic anovulation, obesity, and hyperinsulinemia, resulting in progesterone deficiency. Consequently, the endometrium tends to remain in an oestrogen dominant proliferative state, increasing the risk of developing uterine cancer [158, 159]. Furthermore, metabolic impairment in PCOS consists in insulin resistance accompanied by an increase of IGF-I, both playing a pivotal role in the endometrial cell proliferation and differentiation, promoting the development of EC [160, 161] as follows.

Speculated Pathogenesis of Endometrial Cancer in PCOS Women (Mod from Gadducci et al. [132])

Chronic anovulation → unopposed estrogen stimulation

↑ serum LH
↑ endometrial LH receptor expression

Insulin resistance → chronic hyperinsulinemia

↑ serum IGF-1

The estimated risk of PCOS women in developing endometrial cancer has been evaluated in some observational case-series, case-control studies, systematic reviews, and meta-analysis as well. With only a few exceptions [162], the majority of such studies [144, 159, 163–166] confirmed a higher EC risk for PCOS women. In the early 90s, one of the first case-control studies [167] managed by the National Cancer Institute program of the Surveillance Epidemiology and End Result, assigned to PCOS women an odd ratio (OR) for endometrial cancer of 2.7 compared to the control group. In a further retrospective study, Wild et al. [163] evaluated the long-term endometrial consequences in PCOS women reporting, over 30 years of follow-up, a higher OR (up to 5.3) for developing EC. A systematic review and a meta-analysis by Chittenden et al. [166] explored the long-term outcome of PCOS patients. Among four observational studies, they reported that women with PCOS appear to be three times more likely to develop well-differentiated endometrial cancer. More recently Haoula et al. [159] performed a meta-analysis comparing previous observational studies and confirmed that the risk of developing EC is three times more likely compared with general population. Furthermore, Barry et al. [144] in a case-control study have reported a significantly increased OR of 2.79 for endometrial cancer in women with PCOS and endometrial hyperplasia. Moreover when the meta-analysis was restricted to women younger than 54 years, the risk estimate further increased (OR of 4.05). In a large Danish cohort study, using the National Patient and Cancer Registry [146], during the period 1977–2012, the incidence of endometrial cancer was significantly higher in PCOS-affected women less than 50 years of age, with a standardized incidence ratio of 3.9. Most of these cases were type I endometrial cancer, a finding that supports the pathogenetic mechanism of long-term exposure to unopposed oestrogen.

Finally, a high-scale nationwide population-based cohort study, conducted in Taiwan [145], retrospectively estimated the risk for EC of 8.42 times higher for women with PCOS than for the comparison cohort. All these lines of evidence suggest, as stated by the 3rd PCOS Consensus Group [168], that there are moderate-quality data supporting the high risk of PCOS women for developing endometrial cancer, with consequent need for increased awareness for surveillance strategies. As metformin increases signaling by the insulin receptor, leading to an improvement in insulin resistance and a reduction in circulating insulin levels, it is regularly used as an insulin sensitizer. Furthermore, metformin plays an essential role in the inhibition of hepatic gluconeogenesis [169] with the consequent decrease in insulin levels. Thus, metformin is a cornerstone in treatment of PCOS women for improving reproductive abnormalities, restoring ovulation, and improving fertility [170, 171]. The recognition of PCOS as a risk factor for endometrial cancer has some, although still debated, therapeutic implications. Progesterone and its analogues, as well as a levonorgestrel-releasing intrauterine device, are effectively used to inhibit oestrogen-induced endometrial proliferation while promoting synchronous growth, development, and shedding of a structurally stable endometrium. However, it has been documented that up to 30% of PCOS women with endometrial hyperplasia do not correctly respond to this type of hormonal approach [172, 173], possibly due to a progesterone-resistance, which in turn determines persistence of endometrial hyperplasia

Table 1: Metformin and endometrial proliferative pathology.

Author	Ref	Year	Study	Results
Session et al.	[10]	2003	Human clinical study	MTF regresses atypical endometrial hyperplasia in one patient
Legro et al.	[11]	2007	Human clinical study	MTF resolved simple hyperplasia in two patients
Shen et al.	[12]	2008	Human clinical study	MTF regresses atypical endometrial hyperplasia in two women
Cantrell et al.	[13]	2010	Endometrial cell line experimental	MTF is a potent inhibitor of cell proliferation in endometrial cancer cell lines
Tan et al.	[14]	2011	Human experimental study	MTF reduces *in vitro* endometrial invasion
Li et al.	[15]	2014	Human clinical study	MTF reverts stage IA endometrial carcinoma into normal in five young PCOS patients

MTF: metformin.

and further possible cancer transformation. Such hormonal-resistance may be defined as the clinical failure after high-dose progesterone treatment for 3 months, resulting in the acceleration of atypical EH [14]. To overcome this resistance, metformin therapy may be used in PCOS women affected by proliferative endometrial pathologies. Glucose metabolism in PCOS women and reversing endometrial proliferation as well as modulation of insulin sensitivity are functions of metformin very commonly described. Indeed, an endometrial cancer model has described that exposure of EC cells to sera from PCOS under metformin therapy reduces cell growth, altering signaling pathways involving tumour invasion [14]. Thus, a pivotal biological pathway leading to endometrial cancer may be theoretically controlled by metformin, to reduce the risk of EH progression [174]. Given this background, metformin therapy, with/without progesterone-based oral contraceptives, has been adopted in small anecdotal studies to reverse atypical EH or early-stage EC as well (Table 1). In an early case-report, following the failure of progestogen therapy, Session et al. [10] reported EH regression after metformin. Some year later, Shen et al. [12] confirmed metformin and progestin-based contraceptives as being effective therapeutic combination in two women affected by atypical EH with progestin-resistance.

However, in regard to endometrial cancer outcomes, data concerning a protective efficacy of metformin are still insufficient [157]. In a large retrospective cohort study [175], the use of metformin among diabetic patients with nonendometrioid EC was associated with a statistically improved overall survival (OS). Similarly, in another retrospective cohort analysis EC diabetic patients using metformin had an improved recurrence-free survival and OS than those who did not use metformin [176]. Conversely, other results from observational case-control analysis [177] did not confirm a decreased risk of endometrial cancer in metformin users. In addition to this, due to the lack of studies, a systematic review carried out using the Cochrane Library database [178] did not reach any firm conclusion about a preventive role of metformin in endometrial cancer. Some data support that metformin treatment, for 6 months, results in decreased incidence, progression, and even cancer-related mortality with regard to endometrial cancer. Li et al. [15] described the efficacy of a combination strategy with hormonal therapy (cyproterone acetate and ethinyl estradiol, Diane) and metformin in five PCOS women affected by stage IA endometrial cancer. After 6 months, all of the women reverted to normal endometrium. Even though it was suggested that metformin might become an important pharmacological asset in early-stage endometrial cancer [54], further prospective investigations are needed. For this reason, the North Carolina Lineberger Comprehensive Cancer Center started a pilot study to evaluate the effectiveness of metformin in reverse endometrial hyperplasia. Currently the study is still ongoing, recruiting participants, with an estimated study completion date in December 2018 [179]. Furthermore, at the same Cancer Center, another study is in progress to assess the conservative treatment of EH, with oral progestin or levonorgestrel-intrauterine device (LNG-IUS) plus metformin therapy [180].

6. Conclusions

Several preclinical cancer models, including studies on endometrial cancers cell lines, have shown that insulin-sensitizers might act as anticancer agents in ovarian and endometrial cancers and their ability to act on a variety of pathways, with wide-ranging effects, should be taken into consideration. Although a lot of evidences support the potential role of insulin-sensitizers in prevention and treatment of certain gynaecological cancer, further research efforts are required to confirm their clinical usefulness. In fact, it has been demonstrated that they activate AMPK, a potent inhibitor of the PI3K/Akt/mTOR pathway, prompt G1 cell cycle arrest, induce apoptosis, and decrease human telomerase reverse transcriptase expression. Furthermore, they seem to inhibit mTOR through AMPK-independent pathways, which interfere with the development and DNA damage response mechanisms. Insulin-sensitizers antiangiogenetic effects reduce DNA damage and mutation rates offering an explanation for the reduced risk of cancer seen

in metformin users across several epidemiological studies. PCOS seems to be associated with an increased risk for developing epithelial ovarian cancer due to the abnormal hormonal environment with abnormal concentrations of unopposed oestrogen, even if few studies support the fact that the association between them is limited. Androgens such as testosterone can enhance the growth of ovarian epithelial tumours in animal models and may promote ovarian cancer progression by decreasing TGFβ receptor levels, thereby allowing tumour cells to escape TGFβ 1 mediated growth inhibition. Recently a number of proteins overexpressed in both PCOS and ovarian cancer, such as superoxide dismutase, fibrinogen γ, vimentin, calreticulin, malate dehydrogenase, and lamin B2, have been identified, revealing subgroups of women with PCOS and with an increased risk of developing this malignancy. Metformin appears to decrease endometrial cancer incidence, progression, and even mortality, in particular at an early stage. It appears reasonable to assume that insulin-sensitizers drugs, by reducing the carcinogenic effects of obesity and insulin resistance, might be employed for long-term chemoprevention in women at high risk of endometrial cancer. It is necessary to carry out new retrospective epidemiological research and meta-analysis on wide population of PCOS patients organized in groups based on reproductive axis function, hormonal and metabolic profile, and drugs therapies. Therefore, even though tumoural cell cultures and mouse models have been used to explain insulin-sensitizers' mechanism of action and their potential inhibitory effect on tumourigenesis, new and more physiologically relevant *in vitro* human models are needed to fully elucidate the molecular mechanisms exploited by these drugs and shape clinical studies. Additional research would be useful to better define cytokines and growth factors that are activated in gynaecological cancers and may modulate cellular responses to insulin-sensitizers in women suffering from PCOS. This is an important key point in identifying the most suitable patients to be treated with these drugs and plan adequate clinical trials.

Abbreviations

ACC: Acetyl-CoA Carboxylase
Akt/PKB: Protein kinase B
AMP: Adenosine monophosphate
AMPK: 5′ Adenosine monophosphate-activated protein kinase
ATP: Adenosine-5′-triphosphate
BAD: Bcl2-Antagonist of cell Death
Ca^{2+}: Calcium ions
CDP-DAG: Cytidine diphosphate diacylglycerol
CaMKK: Calcium/calmodulin-dependent protein kinase
COX-2: Cyclooxygenase 2
CYP17: Cytochrome P-45017
DAG: Diacylglycerol
D-chiro-Ins: D-Chiro-inositol
EC: Endometrial cancer
EGF: Epidermal growth factor
EH: Endometrial hyperplasia
EMT: Epithelial to mesenchymal transition
ERK: Extracellular-signaling regulated protein kinase
4E-BP1: Eukaryotic Translation Initiation Factor 4E-Binding Protein 1
FFA: Free fatty acids
GLUT: Glucose transporter
GPCR: G-protein coupled receptor
GSK3β: Glycogen synthase kinase 3
HIF: Hypoxia inducible factor
HMIT: Proton coupled inositol transporters
11β-HSD: 11β-Hydroxysteroid dehydrogenase
3β-HSD: 3β-Hydroxysteroid-dehydrogenase
IGF-I: Insulin growth factor 1
IGF-II: Insulin growth factor 2
IL-6: Interleukin 6
IR: Insulin receptor
Ins(1,4,5)P3: Inositol (1,4,5)-triphosphate
InsP6: Inositol hexaphosphate
IPMK: Inositol polyphosphate multikinase
IRSs: Insulin receptor substrates
ISF-1: Insulin sensitivity factor 1
JNK: c-Jun N-terminal Kinase
K-Ras V-Ki-ras2: Kirsten rat sarcoma viral oncogene homolog
LH: Luteinizing hormone
LKB1: Liver kinase 1
MAPK: Mitogen active proteins kinase
MIPS: 1L-Myoinositol-1-phosphate synthase
MO25: Mouse protein 25
myo-Ins: Myoinositol
MT19c: Nonhypercalcemic vitamin-D2 derived anticancer agent
mTOR: Mammalian target of rapamycin
mTORC1: Mammalian target of rapamycin complex 1
MTF: Metformin
NF-κB: Nuclear factor κB
OCT1: Organic Cation Transporter 1
OR: Odd ratio
p53: Tumour Protein 53
PCOS: Polycystic ovary syndrome
PDK1: Phosphoinositide dependent protein kinase-1
PI: Phosphatidylinositol
PI(4,5)P2: Phosphatidylinositol 4,5-biphosphate
PI(3,4,5)P3: Phosphatidylinositol 3,4,5-trisphosphate
PI3K: Phosphatidylinositol-3-kinase
PIP: Phosphatidylinositol phosphate
PKC: Protein kinase C
PLCγ: Phospholipase C γ
PPARγ: Peroxisome proliferator-activated receptor gamma
PRAS40: Proline-Rich Akt/PKB Substrate 40 kDa
PTEN: Phosphatase and tensin homolog
RAC1: Ras-Related C3 Botulinum Toxin Substrate 1
REDD1: Regulated in development and DNA damage responses 1
RHEB: Ras Homolog Enriched in Brain
RPTOR: Regulatory-associated protein of mTOR
RR: Relative risk

RXR: Retinoid X receptor
S6K1: Ribosomal protein S6 kinase 1
TAK1: Transforming growth factor β-activated kinase 1
SHBG: Sex hormone binding globulin
SMIT1/2: Sodium-dependent myoinositol transporters 1 and 2
STRAD: STe20-Related Adaptor
TGFβ: Transforming growth factor β
TNFα: Tumour Necrosis Factor α
TSC2: Tuberous sclerosis complex-2
TZDs: Thiazolidinediones
VEGF: Vascular endothelial growth factor.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Authors' Contributions

Rosa Lauretta and Giulia Lanzolla are authors with equal contribution.

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Research Article

Effect on Insulin-Stimulated Release of D-Chiro-Inositol-Containing Inositolphosphoglycan Mediator during Weight Loss in Obese Women with and without Polycystic Ovary Syndrome

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Background. A deficiency of D-chiro-inositol-inositolphosphoglycan mediator (DCI-IPG) may contribute to insulin resistance in polycystic ovary syndrome (PCOS). Whether the relationship between impaired DCI-IPG release and insulin resistance is specific to PCOS rather than obesity is unknown. We assessed insulin-released DCI-IPG and its relationship to insulin sensitivity at baseline and after weight loss in obese women with and without PCOS. *Methods.* Obese PCOS ($n = 16$) and normal ($n = 15$) women underwent 8 weeks of a hypocaloric diet. The Matsuda index, area under the curve DCI-IPG ($AUC_{DCI\text{-}IPG}$), $AUC_{insulin}$, and $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ were measured during a 2 hr OGTT at baseline and 8 weeks. *Results.* PCOS women had lower $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ at baseline and a significant relationship between $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ and Matsuda index ($p = 0.0003$), which was not present in controls. Weight loss was similar between PCOS (−4.08 kg) and normal women (−4.29 kg, $p = 0.6281$). Weight loss in PCOS women did not change the relationship between $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ and Matsuda index ($p = 0.0100$), and this relationship remained absent in control women. *Conclusion.* The association between $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ and insulin sensitivity was only found in PCOS but not in normal women, and this relationship was unaffected by weight loss. DCI and its messenger may contribute to insulin resistance in PCOS independent of obesity.

1. Introduction

Polycystic ovary syndrome (PCOS) affects approximately 6–10% of women of reproductive age [1]. The disorder is characterized by chronic oligo- or anovulation and biochemical or clinical androgen excess. PCOS is also associated with increased risk for diabetes, metabolic syndrome, and early cardiovascular disease. Insulin resistance and its compensatory hyperinsulinemia play a central role in the pathogenesis of PCOS [2, 3]. Multiple lines of evidence indicate that a putative inositolphosphoglycan (IPG) second messenger, D-chiro-inositol-inositolphosphoglycan mediator (DCI-IPG), may mediate insulin action [4]. A deficiency of DCI-IPG may contribute to insulin resistance in individuals with Type 2 diabetes [5] as well as women with PCOS [6, 7]. Interventional studies with oral administration of DCI reported decreases in serum insulin and androgen levels, as well as improved ovulatory function in obese women with PCOS [8–10]. Conversely, administration of insulin sensitizers such as metformin [7] and pioglitazone [11] also increases

insulin-stimulated release of DCI-IPG in women with PCOS.

Our group has previously demonstrated that the coupling between insulin action and release of DCI-IPG mediator is impaired in *obese* women with PCOS, as compared to *nonobese* normal women, suggesting that the insulin-stimulated release of bioactive DCI-IPG mediator is defective in PCOS women with obesity [12]. However, whether the relationship between impaired DCI-IPG mediator release and insulin resistance is specific to PCOS status or due to obesity per se is unknown. We hypothesize that the insulin-stimulated DCI-IPG mediator modulates insulin sensitivity in women with PCOS but not in normal women, and a reduction in obesity does not affect this relationship. To test this hypothesis, we conducted a pilot weight loss intervention study in obese women with PCOS and obese normal women. We assessed the release of both insulin and DCI-IPG mediator during an oral glucose tolerance test (OGTT), as well as insulin sensitivity as measured by the Matsuda index, at baseline and after 8 weeks of a hypocaloric diet in obese women with and without PCOS.

2. Materials and Methods

2.1. Participants. This study was performed at the Clinical Research Service Unit of Virginia Commonwealth University's Center for Clinical and Translational Research. The study was approved by the Virginia Commonwealth University Institutional Review Board. All study participants provided informed consent.

Women in this study were obese ($\geq$30 kg/m^2) and between the ages of 18 and 40 years. PCOS was defined by the modified Rotterdam criteria, after excluding other endocrine disorders [13]. In this study, all PCOS women had biochemical hyperandrogenemia and oligo- or amenorrhea (eight or few menstrual periods annually). Secondary causes of hyperandrogenemia or ovulatory dysfunction were excluded by normal thyroid function tests and serum prolactin and a fasting 17α-hydroxyprogesterone <200 ng/dL. The control group consisted of regular cycling women with normal serum testosterone. The exclusion criteria for all women included weight loss attempts by either diet or exercise within 3 months of study participation, diabetes mellitus by fasting glucose or oral glucose tolerance test (OGTT), clinically significant pulmonary, cardiac, renal, hepatic, neurologic, psychiatric, infectious, neoplastic, and malignant disease, or pregnancy as documented by urine hCG. PCOS women with disorders associated with insulin resistance, for example, hypertension or dyslipidemia, were not excluded as long as they had been on a stable dose of medication for 6 months. Normal women were excluded if they had a history of gestational diabetes or had a first-degree relative with diabetes or if they demonstrated abnormal glucose tolerance at baseline or if they had hypertension or dyslipidemia.

2.2. Study Procedures. PCOS women were studied during the equivalent of the follicular phase of the cycle, and normal women were studied during the mid-follicular phase of the menstrual cycle (days 5–9), as documented by a serum progesterone $\leq$2 ng/mL.

Because DCI may be ingested in a diet high in legumes or fruits, all subjects were interviewed by a dietician to identify those who may be consuming diets containing unusually high amounts of inositols. All participants were given instructions for a balanced mixed diet to be followed for at least three days prior to each study visit.

On the study day, the participants arrived at the Clinical Research Service Unit at Virginia Commonwealth University at 08:00 h after a 12-hour fast. Height and weight were measured to the nearest 0.1 cm and 0.1 kg using a precision stadiometer and digital scale. Waist was measured at the level of the umbilicus, and hip circumference was measured at the widest diameter of the buttocks to the nearest 0.1 cm. Fasting blood samples were drawn at 08:15, 08:30, and 08:45 h and pooled for determination of fasting insulin, glucose, and sex steroids (testosterone). At 09:00 h, an OGTT was performed by administering 75 g oral glucose. Blood samples for plasma glucose, insulin, and DCI-IPG were collected every 15 minutes for 2 hours.

After glucose and DCI assessments, the participants met with a study dietician for instruction on a hypocaloric diet. A diet-overview handout, instructional nutrition labels, sample menus and recipes, and a book on counting calories were provided. The women were instructed to follow an 8-week course of standardized hypocaloric diet containing 50% carbohydrates, 30% total lipids, and 20% proteins. They were instructed to maintain these hypocaloric diets by caloric restriction to create a deficit of 500–1000 kcal/day, as per obesity management guidelines of the National Heart, Lung, and Blood Institute [14]. This hypocaloric diet has been shown to yield weight loss of about 1 to 2 lbs/week [14]. The women were instructed specifically to avoid making any conscious effort to modify physical activity or attempt other weight loss methods in addition to the hypocaloric diets per this protocol. This is because physical activity improves insulin sensitivity even in the absence of substantial weight loss [15] and will confound our investigation of the effect of weight reduction in DCI handling and insulin sensitivity in these women. During this 8-week period, the participants purchased and prepared their own meals and maintained daily food logs. They attended follow-up visits once weekly for weight measurements. During these weekly visits, they submitted their food logs and received follow-up consultations with the study dietician.

The women returned for DCI and insulin sensitivity measurements after 8 weeks of dietary intervention. After confirmation that they were in the equivalent of the follicular phase of the menstrual cycle by serum progesterone, all measurements and testing performed at baseline (anthropometric measurements, OGTT, and blood sampling) were repeated.

2.3. Laboratory Analyses. Serum and plasma were stored at −80°C until being assayed. Serum glucose was measured by glucose oxidative method (YSI 2300 Stat Plus Glucose Analyzer; Yellow Springs Instruments). Serum insulin levels were measured by enzyme-linked immunosorbent assay (ELISA)

(Alpco Diagnostics, Salem NH). Serum testosterone and sex hormone binding globulin (SHBG) were measured via ELISA (Alpco Diagnostics). Free testosterone was calculated using the method of Södergard et al. [16]. DCI-IPG bioactivity was measured using an in-house bioactivity assay developed by the laboratory of JEN, as previously described [7].

2.4. Statistical Analysis. We examined the response of serum insulin concentrations and the relative bioactivity of DCI-IPG to the oral administration of glucose by calculating the areas under the respective response curves (AUC) by the trapezoidal rule. Since insulin is thought to mediate the release of DCI-IPG after a glucose load [17] and there are interparticipant variations in $AUC_{insulin}$, the ratio of $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ more accurately reflects insulin-mediated release of DCI-IPG than $AUC_{DCI\text{-}IPG}$ alone. Hence, we used this ratio in our analyses. Whole body insulin sensitivity as described by Matsuda and DeFronzo [18] was used to assess insulin sensitivity.

Comparisons between groups at baseline were made with Student's two-tailed t-test. To assess within group effects from baseline to after treatment, a matched pairs two-tailed t-test was performed. To assess the treatment effects between groups, the changes in each variable (after weight loss minus baseline) were compared using a two-tailed t-test. Pearson's correlation was used to assess the association between change in Matsuda index and change in bioactive DCI-IPG released per unit of insulin during OGTT, after linearity and normality of residuals were assessed.

Distribution of the data was assessed by normal quantile plots. Variables not in normal distribution were log-transformed for analyses and then backtransformed into their original units for reporting. Data were presented as mean ± standard deviation or geometric mean (95% confidence interval [CI]) for parameters that were transformed for analyses. $p < 0.05$ was considered statistically significant. Analyses were performed by JMP 12.0 (SAS Institute, NC).

3. Results

A total of 80 women provided consent to participate. Of these, 19 met exclusion criteria before study entry. Of the remaining 34 PCOS and 27 normal women, 18 PCOS and 12 normal women dropped out prior to the follow-up visit. Hence, 16 PCOS and 15 normal women completed the study. The attrition rate in this study was similar to that of other dietary-based weight loss studies [19]. Because the purpose of this study is to evaluate the relationship between changes in DCI-IPG mediator release and changes in insulin sensitivity during weight loss in PCOS as compared to normal women, we only included women who completed the study.

3.1. Baseline Characteristics. At baseline, control women and women with PCOS did not differ in terms of age, racial mix, BMI, or waist-to-hip ratio (Table 1). As expected, PCOS women tended to have significantly higher serum total testosterone. Although women with PCOS had higher $AUC_{glucose}$ and $AUC_{insulin}$ and lower whole body insulin sensitivity as determined by Matsuda index, these differences did not attain statistical significance.

Table 1: Baseline characteristics and serum hormone concentrations.

Parameter	PCOS ($n = 16$)	Control ($n = 15$)	p value
Age (years)	26.9 (4.6)	27.5 (5.7)	0.7267
Race			0.5649
African-American	7	7	
Caucasian	8	7	
Other	1	1	
Weight (kg)	99.2 (13.3)	97.6 (15.4)	0.7508
BMI (kg/m^2)	36.6 (5.1)	35.8 (4.8)	0.6507
Waist circumference (cm)	101.6 (12.7)	99.9 (9.9)	0.6860
Waist/hip ratio	0.82 (0.05)	0.80 (0.08)	0.4102
Total testosterone (ng/dL)	63.1 (34.4)	27.7 (8.0)	0.0006
Free testosterone (ng/dL)	0.90 (0.87)	0.44 (0.25)	0.0786
Fasting insulin (mg/dL)*	7.58 (5.65–10.15)	8.12 (5.06–13.03)	0.7897
Fasting glucose (mg/dL)	85.5 (8.1)	84.2 (3.8)	0.6000
AUC insulin (min·mg/dL)	7999 (5058)	5667 (2890)	0.1323
AUC glucose (min·mg/dL)	14781 (3016)	12963 (2245)	0.0715
Matsuda index	5.22 (2.18)	6.94 (4.06)	0.1577
AUC DCI-IPG (% min)	15279 (6030)	21441 (19376)	0.2539
Ratio of AUC DCI-IPG/AUC insulin (%/μIU/mL)*	2.76 (1.79–3.73)	4.83 (2.37–7.29)	0.0377

Values are mean (SD) or geometric mean (95% confidence interval) when indicated by *.

At baseline, women with PCOS had significantly lower $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ ratios. In PCOS women, there was a significant relationship between $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ and Matsuda index ($r = 0.8065$, $p = 0.0003$, Figure 1(a)). This relationship was not found in control women ($r = 0.1488$, $p = 0.6445$, Figure 1(b)).

3.2. Changes in Insulin, Glucose, and the Bioactivity Profiles of DCI-IPG after Weight Loss. After the weight loss intervention, both PCOS (-4.08 ± 3.65 kg, $p = 0.0013$) and control women (-4.69 ± 2.98 kg, $p = 0.0005$) lost weight compared to baseline. The amount of weight loss did not differ between the groups ($p = 0.6281$) (Table 2). However, the Matsuda index improved significantly only in normal women (from 6.94 ± 4.06 to 9.53 ± 4.79, $p = 0.0479$) but not in PCOS women (from 5.22 ± 2.18 to 5.39 ± 2.52, $p = 0.8209$). Weight loss did not significantly increase $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ from baseline in either group ($p = 0.6387$ in PCOS and $p = 0.9697$ in normal women).

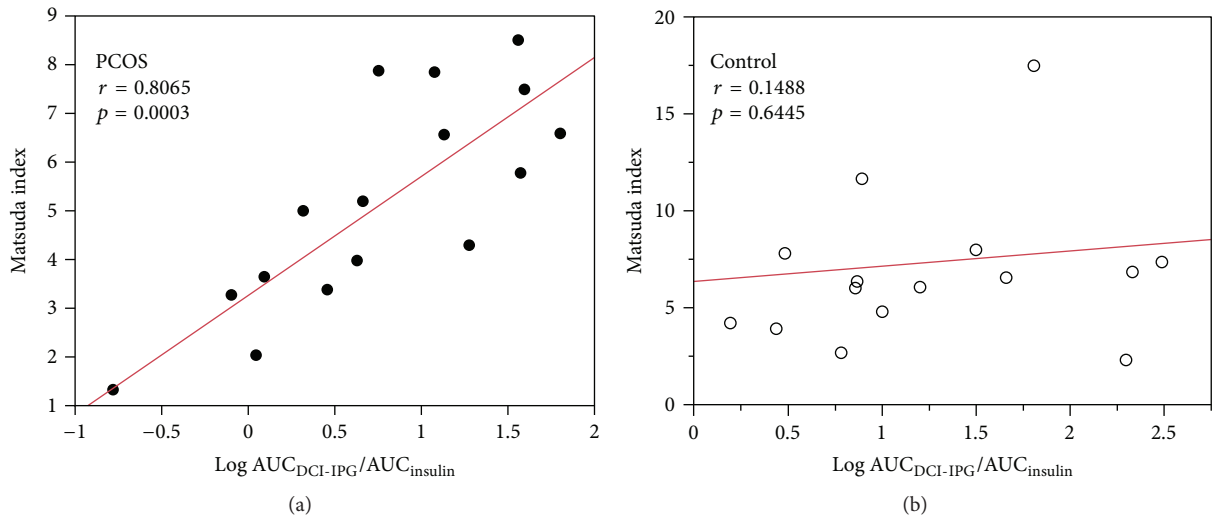

Figure 1: Relationship between baseline Matsuda index and release of the bioactive DCI-IPG messenger per unit of insulin released during OGTT in PCOS (●, a) and normal (○, b) women. DCI-IPG, D-chiro-inositol-inositolphosphoglycan mediator.

Table 2: Changes in metabolic parameters after 8 weeks of weight loss intervention in women with and without PCOS.

Parameter	PCOS ($n = 16$)	Control ($n = 15$)	p value
Weight (kg)	−4.08 (−2.13, −6.02)[a]	−4.69 (−2.89, −6.49)[a]	0.6281
BMI (kg/m^2)	−1.46 (−0.72, −2.20)[a]	−1.80 (−1.14, −2.45)[a]	0.4829
Waist/hip ratio	−0.014 (−0.034, 0.006)	−0.040 (−0.107, 0.027)	0.3878
Fasting insulin (mg/dL)	+1.8 (−2.2, +5.7)	−5.0 (−10.6, +0.6)	0.0625
Fasting glucose (mg/dL)	−1.15 (−4.43, +2.13)	+0.21 (−2.35, +2.77)	0.5041
AUC insulin (min·mg/dL)	−1009 (−2601, +642)	−1403 (−2886, −80)[b]	0.1961
AUC glucose (min·mg/dL)	−961 (−1944, +21)	+152.8 (−1245, +1550)	0.1600
Matsuda index	+0.17 (−0.87, +1.21)	+2.60 (+0.38, +5.58)[c]	0.1168
AUC DCI-IPG (% min)	−446 (−4078, +3185)	−3754 (−11496, +3989)	0.6765
Ratio of AUC DCI-IPG/AUC insulin (%/μIU/mL)	+1.085 (−0.709, 1.663)	+1.049 (−0.798, 1.380)	0.8805

Data are expressed as mean (95% confidence interval).
[a] $p < 0.002$ for within group difference between baseline and after weight loss.
[b] $p = 0.0134$ for within group difference between baseline and after weight loss.
[c] $p = 0.0479$ for within group difference between baseline and after weight loss.

3.3. Relationship between DCI-IPG Mediator Bioactivity and Insulin Sensitivity after Weight Loss. Weight loss did not change the relationship between $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ and Matsuda index in PCOS women. Among women with PCOS, after weight loss there remained a significant relationship between change in $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ and change in Matsuda index ($r = 0.6412$, $p = 0.0100$, Figure 2(a)). This relationship was not found in control women ($r = 0.2717$, $p = 0.3928$, Figure 2(b)).

4. Discussion

In this study, we observed that obese women with PCOS, as compared to normal women with similar BMI, have decreased insulin-released DCI-IPG mediator during an OGTT. We observed that the relationship between insulin sensitivity as measured by the Matsuda index and $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ was found only in obese women with PCOS and not in obese normal women. Furthermore, this relationship was unaffected by weight loss. After a similar amount of weight loss, a significant relationship between $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ and Matsuda index remained only in women with PCOS but was not present in normal women.

The findings of our study are in concordance with our previous report of significantly lower $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ ratios in PCOS women as compared to normal women [6]. However, in the previous study, PCOS participants had a significantly higher BMI (33.9 kg/m^2) than normal women (25.6 kg/m^2, $p = 0.002$). Our current study demonstrates that, even with similar obesity, $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ remained lower in PCOS women compared to normal women ($p = 0.0377$, Table 1). Our findings suggest that bioactivity of the DCI-IPG mediator is decreased in PCOS independent of obesity.

We also observed that the relationship between insulin sensitivity and $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ was present only in women with PCOS (Figure 1(a)) and not in normal women (Figure 1(b)) and that this finding remained evident after weight loss (Figures 2(a) and 2(b)). These results are supported by our previous findings of a significant association

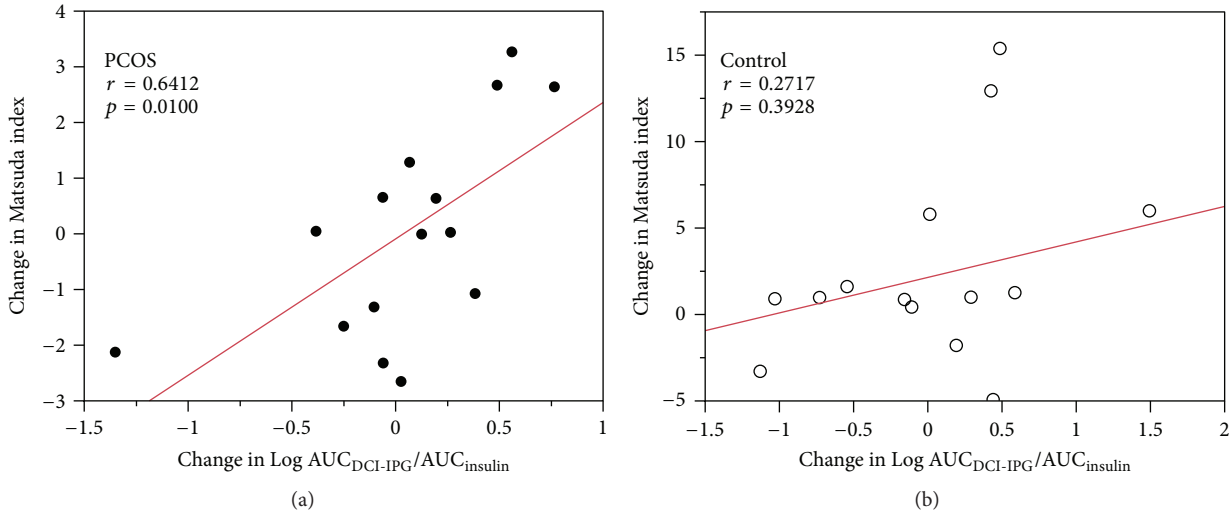

Figure 2: Relationship between change in Matsuda index and change in release of the bioactive DCI-IPG messenger per unit of insulin released during OGTT in PCOS (●, a) and normal (○, b) women after 8 weeks of weight loss intervention. DCI-IPG, D-chiro-inositol-inositolphosphoglycan mediator.

between change in insulin sensitivity and change in DCI-IPG released per unit of insulin with oral DCI administration in PCOS women [20]. However, previous studies only examined this association in women with PCOS, and whether $AUC_{DCI-IPG}/AUC_{insulin}$ is correlated with insulin sensitivity in normal women with similar BMI has been unknown. To the authors' knowledge, this is the first report suggesting that DCI-IPG mediator release may not play a major role in insulin sensitivity in normal obese women.

Weight loss did not affect the relationship between insulin sensitivity and $AUC_{DCI-IPG}/AUC_{insulin}$ in obese women with PCOS in this study. This finding is in line with our previous reports supporting that DCI deficiency in PCOS may be unrelated to adiposity. To wit, administration of oral DCI improved $AUC_{insulin}$, serum androgens, and ovulation to both obese [8] and lean [9] women with PCOS.

In this current study, weight loss did not significantly improve $AUC_{DCI-IPG}/AUC_{insulin}$ in either PCOS or normal women. In contrast, previous studies demonstrated that insulin sensitizers such as metformin [7] and rosiglitazone [11] improved the availability of DCI-IPG mediator release in women with PCOS.

Why do insulin sensitizers, but not weight loss as described in this study, improve the $AUC_{DCI-IPG}/AUC_{insulin}$ bioactivity profile in women with PCOS? One reason could be that weight loss of more than 4 kg in this study did not improve insulin sensitivity in obese women with PCOS. At first glance, our results seem to contradict previous research supporting the role of weight loss in improving insulin sensitivity in PCOS [21]. However, there is tremendous heterogeneity in the effect of weight loss on improving insulin sensitivity and other features of PCOS [22, 23]. In one weight loss study, as many as 50% of PCOS women did not have improved insulin sensitivity as measured by HOMA and, commensurably, no improvement in menstrual cyclicity, despite similar fat losses in both responders and nonresponders [23].

We did not observe a difference in the amount of weight loss between women with and without PCOS in this study. There have been conflicting reports about the role of insulin resistance in the regulation of obesity. Some studies suggested insulin resistance predicted weight gain [24], more weight loss [25], or no effect on weight loss [26] in obese individuals. Hence, the knowledge that weight loss is not different between PCOS and normal women when given the same hypocaloric diet can be reassuring to women with PCOS who are attempting weight loss.

A strength of this study includes similar BMI between PCOS and normal women, which helped elucidate that the relationship between $AUC_{DCI-IPG}/AUC_{insulin}$ bioactivity and insulin sensitivity is specific to PCOS and not obesity. These results are novel since the roles of DCI-IPG mediator in normal obese women have not been previously explored.

A weakness of the study is that the amount of weight loss achieved in both groups of women may have been inadequate to illicit changes in DCI-IPG/insulin ratio. Although the amount of weight loss (0.5 kg or approximately 1 lb per week) was in accordance with current weight management guidelines [14], over the course of the 8-week study period, it resulted in a reduction in weight by about 4 kg in both groups, which was less than that achieved in other weight loss studies in PCOS [23, 27]. A study with a longer duration would have resulted in a bigger magnitude in weight reduction.

In conclusion, this study demonstrated that obese women with PCOS, as compared to normal women with similar BMI, have decreased insulin-released DCI-IPG mediator during OGTT. The relationship between insulin sensitivity and $AUC_{DCI-IPG}/AUC_{insulin}$ is only found in women with PCOS but not in normal women. Furthermore, this relationship is unaffected by weight loss. After a similar amount of

weight loss, a significant relationship between $AUC_{DCI\text{-}IPG}/AUC_{insulin}$ and Matsuda index is only found in women with PCOS but not in normal women. Combined with previous studies of oral DCI administration in PCOS women by our group and others, this study reinforces the contribution of DCI and its messenger in its role in insulin resistance in women with PCOS independent of obesity.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

In Vitro Antioxidant Treatment of Semen Samples in Assisted Reproductive Technology: Effects of Myo-Inositol on Nemaspermic Parameters

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Male infertility and the poor quality of sperm seem to be influenced by oxidative stress. In particular, the reactive oxygen species (ROS) mainly produced by morphologically altered spermatozoa affect sperm motility, morphology, and integrity. The aim of this study was to evaluate the efficacy of Myo-Inositol (Myo-Ins) on a number of parameters such as viscosity and total and progressive motility of spermatozoa, in order to better validate its possible practical application *in vitro*, in order to improve the capacitation protocols commonly used in Assisted Reproductive Technology (ART). A total of 100 fresh and 25 thawed semen samples were analyzed *in vitro* prior to and after addition of Myo-Ins. Treatment of samples with Myo-Ins showed an increase in the sperm total and progressive motility in both fresh and thawed samples. Furthermore, Myo-Ins proved to be well tolerated by spermatozoa *in vitro*, demonstrating that it can be efficiently and safely used as antioxidant in the laboratory practice and for preparation of semen samples in ART.

1. Introduction

Oxidative stress (OS) has been shown to play a crucial role in the pathogenesis of sperm dysfunction and sperm DNA damage in infertile men [1–4]. Spermatozoa are very susceptible to the negative action of reactive oxygen species (ROS), affecting mainly sperm motility, morphology, and integrity. Indeed, spermatozoa dysfunctionality, damaged sperm DNA, and reduced male reproductive potential are caused by high levels of ROS in semen. White blood cells and sperm cells, prematurely released from the seminiferous tubules, seem to be the two main sources of ROS [5, 6]. However, small amounts of hydrogen peroxide or other free radicals, such as nitric oxide and superoxide anion, have been shown to stimulate sperm capacitation and hyperactivation for binding to the zona pellucida and for the acrosome reaction [5–11], suggesting that, after all, ROS, at low concentration, play a key role in sperm functions. During capacitation *in vivo*, higher levels of intracellular Ca^{2+}, ROS, or tyrosine kinase have been found, leading to an increase in cyclic adenosine monophosphate (cAMP), which in turn promotes sperm motility [12]. Only capacitated spermatozoa show adequate motility and undergo the acrosome reaction, thus acquiring fertilizing capacity [8]. Despite the physiological role played by free radicals, spermatozoa are also subjected to the delicate balance between free radicals and antioxidant barrier, being constantly exposed to the "oxygen paradox": oxygen and its metabolites at low levels are essential for survival and for the maintenance of normal cellular functions but at the same time can impair function and survival [13]. A close relationship between the production of free radicals and altered sperm function has been demonstrated by a number of studies, showing that the sperm capability in merging with the zona pellucida is inversely proportional to the production of ROS [5]. This predisposition to the free radicals effect is primarily due to the sperm structure. Human spermatozoa

contain a high concentration of polyunsaturated fatty acids (PUFAs), especially docosahexaenoic acid, that confer fluidity to the plasma membrane, crucial for the fertilization step [7]. The possible presence of transition metals, such as ferrous ions, within the culture media, can promote lipid peroxidation of sperm contributing to a low performance of *in vitro* fertilization (IVF) [14]. ROS production also seems to be strongly linked to sperm morphological quality. Defects in the cytoplasmic extrusion mechanism lead to an excess of residual cytoplasm. These immature sperm and their cytoplasmic excess are responsible for the production of ROS mediated by cytosolic glucose-6-phosphate dehydrogenase (G6PD) [5, 7] according to two possible mechanisms: through the system nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the sperm plasma membrane and the NADPH oxidase-dependent reductase oxide system in mitochondria.

Myo-Inositol (Myo-Ins) is one of the nine stereoisomers of Inositol, a physiological compound belonging to the sugar family; it is found in seeds, whole grains, and fruits as well as in human cell membranes. Myo-Ins, present in cell membranes, is involved in cell growth, lipid synthesis, cell cytogenesis, and morphogenesis. The concentration of Myo-Ins differs throughout the reproductive system, increasing along the epididymis and the vas deferens [15]. Indeed, higher levels of Myo-Ins are found in seminiferous tubule fluid than in seminal plasma. Myo-Ins plays a key role as second messenger by regulating the levels of intracellular Ca^{2+} which in turn regulates sperm motility, capacitation, and acrosome reaction. All these mechanisms occur in spermatozoa at the plasma membrane and mitochondria level. All these findings have led to testing Myo-Ins as a possible antioxidant agent in case of male infertility with either oral administration or *in vitro* use. Indeed, a number of recent studies have shown that Myo-Ins can be used to improve the parameters of semen in patients undergoing ART cycles [16, 17]. Two further studies carried out by Condorelli et al. suggest a possible use of Myo-Ins both *in vivo* and *in vitro* for treatment of male infertility [15, 18]. In particular, a significant increase in the percentage of spermatozoa with high mitochondrial membrane potential (MMP) in oligoasthenoteratozoospermic (OAT) patients was found, leading to increased progressivity and concentration of motile sperm.

Therefore, this might suggest that the use of Myo-Ins for the treatment of male infertility both *in vivo* and *in vitro* may have a positive effect on ART outcomes. Taking into account all these findings, we aimed at evaluating further the role and the efficacy of Myo-Ins on a number of parameters such as viscosity and total and progressive motility of spermatozoa, in order to validate its possible practical application, in order to improve the capacitation protocols already used commonly in ART.

2. Materials and Methods

2.1. Patient Selection. A total of 100 men aged 22–60 years, including 46 normozoospermic subjects, 19 oligozoospermic subjects, 15 asthenozoospermic subjects, and 20 oligoasthenozoospermic subjects, were enrolled in this study. Patients were selected taking into account the evaluation criteria, collected during the preanalytical interview, such as cigarette smoking, testicular surgery, living in areas of environmental risk, and drugs administration (in particular antibiotics) 3 months prior to recruitment. The exclusion criteria included cryptozoospermia, azoospermia, and ejaculate volume less than 1.5 mL. Furthermore, in this study 25 thawed semen samples, from patients aged 28–51 years, were also assessed. Among these samples, cases of severe oligo- and asthenozoospermia, with ejaculate volume of less than 1.5 mL, coming from either biopsy or fresh ejaculate, were analyzed. Specifically, the semen samples were previously collected from 3 normozoospermic, 7 oligozoospermic, 6 asthenozoospermic, and 9 oligoasthenozoospermic subjects. Also in this case anamnestic information from each patient was gathered during the interview.

2.2. Myo-Ins Exposure and Sperm Analyses. Semen samples were freshly collected by masturbation after 3–5 days of sexual abstinence. Each sample was maintained at 37°C for about 20 minutes to allow the liquefaction of the seminal coagulum. In the execution of semen analysis, all the microscopic and macroscopic parameters of the ejaculate were evaluated using as reference values reported in the 2010 edition of the WHO manual [18]. Parameters like sperm concentration and total and progressive motility were carried out within the first hour of ejaculation in order to limit the alterations due to dehydration and pH and temperature changes, using the Makler counting chamber. The capacitation protocols used were swim-up and discontinuous density gradients.

2.3. Preparation and Storage of the "Antioxidant Medium". Five mL of sperm washing/insemination medium (HEPES buffered EBSS, 4 g/L Human Serum Albumin) was enriched with 750 μL Myo-Ins (Andrositol Lab, LO.LI. Pharma, Rome) to obtain a final concentration of 10x; the culture medium thus obtained was stored at cool temperature (between 0° and 25°C) and kept away from direct sources of light.

2.4. Preparation of Fresh and Thawed Semen Samples. The semen samples were prepared according to the following procedure: the day of sample collection, 100 μL Myo-Ins (from stock 10x) was added to an aliquot of 900 μL seminal sample, in order to obtain a final concentration of 1x; semen samples were then carefully pipetted and incubated for 15 minutes at 37°C; at the end of incubation, the viscosity, concentration, and total and progressive motility were assayed with the same procedures adopted for the analysis of the samples. Capacitation was carried out to the untreated samples and those treated with Myo-Ins. The separation of sperm from seminal plasma was performed to obtain a final preparation containing a high percentage of motile cells, free of debris and germ cells. 100 μL of thawed semen sample was mixed either with 24 μL of antioxidant medium prepared, in order to obtain a final concentration of 2x, or with 100 μL of pentoxifylline solution. The semen samples were carefully

pipetted and incubated for 15 minutes at 37°C and assayed as above.

2.5. Statistics. Data are indicated as mean values ± SD. Significance of differences between intragroup comparisons was processed using paired t-test (GraphPad Software, La Jolla, USA). A two-tailed p value < 0.05 value was utilized throughout as a criterion for any result that was statistically significant.

3. Results

Data from the motility is reported as mean percentage of motile spermatozoa of the total spermatozoa. Total sperm motility increased significantly in fresh samples before capacitation after the addition of Myo-Ins from 46.55 ± 18.62% to 50.23 ± 18.92% ($p \leq 0.0001$) (Figure 1). A significant increase was observed also in sperm progressivity before capacitation after treatment with Myo-Ins from 47.76 ± 20.64% to 56.91 ± 20.68% ($p \leq 0.05$). A slight but significant increase was observed in the total sperm motility of fresh samples after capacitation (from 73.99 ± 28.94% to 70.87 ± 31.46%, $p \leq 0.05$), whereas a minor but not significant reduction in the sperm progressive motility after capacitation was observed after Myo-Ins treatment (from 70.67 ± 26.72% to 69.97 ± 27.27%) (Figure 1). The difference of progressive motility in fresh samples is shown in Figure 2: the progressive motility between fresh sample and the sample treated with Myo-Ins showed a difference of 30%. A very small difference was observed between fresh sample after capacitation and sample treated with Myo-Ins after capacitation (1.48%), whereas a higher percentage was observed in fresh sample after capacitation and sample treated with Myo-Ins after capacitation (16.65%). Sperm total motility of thawed samples slightly increased after addition of Myo-Ins, but data was not significant (from 11.4 ± 16.51% to 14.88 ± 16.86%); instead, progressive motility of same samples showed a significant increment after treatment with Myo-Ins (from 9.8 ± 14.1% to 16.4 ± 20.64%, $p \leq 0.05$), (Figure 3). Treatment of fresh semen samples with pentoxifylline did not alter significantly the sperm motility, either the total or the progressive motility (from 9.87 ± 18.26% to 10.93 ± 10.36% and from 7.18 ± 13.9% to 6.875 ± 15.26%, resp.) (Figure 4).

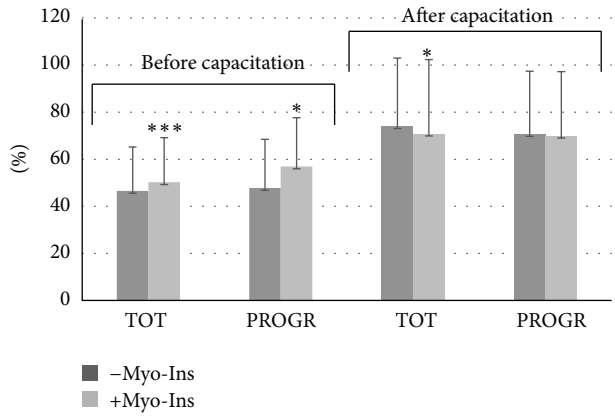

Figure 1: Sperm motility. Values are shown as mean ± SD. Statistical difference between pre- and post-Myo-Ins treatment: $^{*}p \leq 0.05$; $^{***}p \leq 0.0001$.

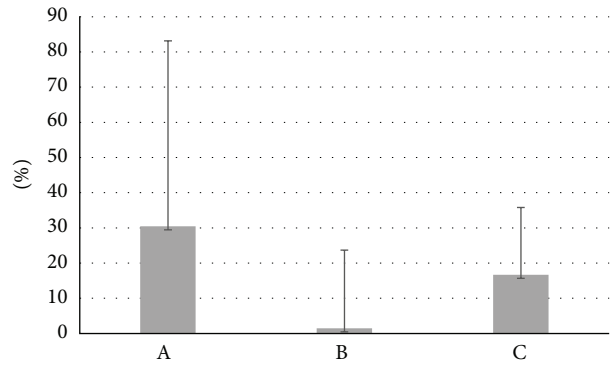

Figure 2: Difference of progressive motility in fresh samples. Values are shown as mean ± SD. A: fresh sample and the sample treated with Myo-Ins. B: fresh sample after capacitation and sample treated with Myo-Ins after capacitation. C: fresh sample after capacitation and sample treated with Myo-Ins after capacitation.

4. Discussion and Conclusions

In this study, the beneficial effect of Myo-Ins *in vitro* in improving sperm total and progressive motility from patients with OAT was shown. Male infertility seems to be a serious clinical problem among men of reproductive age. The causes are still unknown, and about 15% of couples are affected by idiopathic infertility. However, environmental, genetic, psychological, and hormonal factors seem to play a critical role in increasing the incidence of this clinical condition. Although the molecular basis of idiopathic infertility has not been clearly described, OS appears to be one of the main mechanisms involved [19–21]. The link between OS and male infertility has been examined in depth by many researches, suggesting that a high amount of radicals is produced by leukocytes of seminal plasma or by morphologically altered and immature spermatozoa [22–29]. Despite the fact that a minimal quantity of ROS is required for normal sperm functions [13], such as capacitation and the acrosome reaction, their excessive production can lead to loss of sperm integrity as well as activity. Indeed, higher levels of ROS are found in infertile men's semen compared to fertile men. Worldwide approval and interest on the antioxidant therapies *in vivo* are constantly growing, either to enhance the partners' natural fertilizing ability or to increase effectiveness of the assisted reproductive program. A correlation between antioxidants deficiency and male infertility has not been disclosed yet; however, it could be that a subset of men may be at risk of infertility because of the antioxidant shortage [30]. Indeed, OS, among the many causes of male infertility, has been identified as one of the main factors that can deplete the fertilizing potential of sperm and, for this reason, in recent years it has been studied by several research groups. Studies confirm that oral use of antioxidants protects the morphological and functional integrity of sperm from the

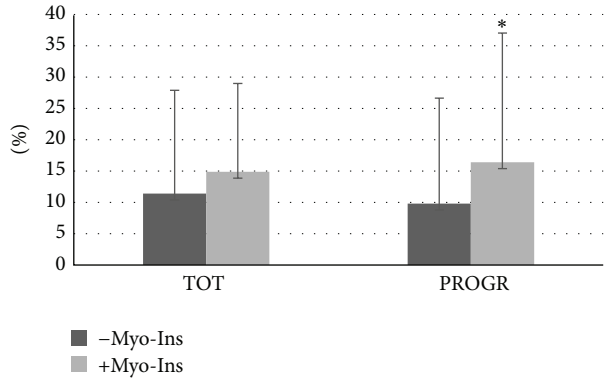

Figure 3: Sperm motility of thawed samples. Values are shown as mean ± SD. Statistical difference between pre- and post-Myo-Ins treatment: $^{*}p \leq 0.05$.

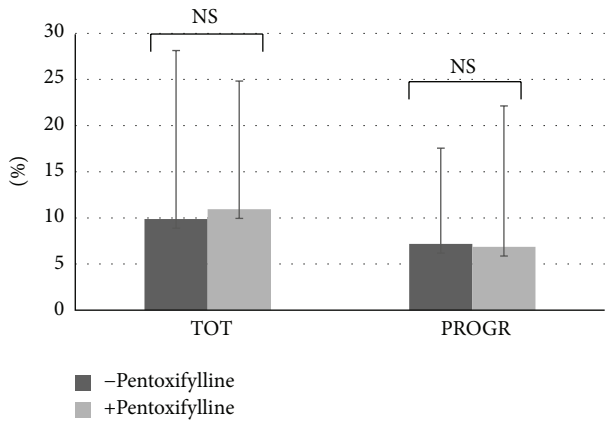

Figure 4: Sperm motility after treatment with pentoxifylline. Values are shown as mean ± SD. Statistical difference between pre- and post-pentoxifylline not significant (NS).

consequent alterations to ROS excesses. Use of antioxidants in the laboratory practice *in vitro* could be useful to optimize certain desired parameters, and it can represent a relevant step for IVF. To date, however, there are very few medical devices on the market; therefore, not much data is available on the direct action *in vitro*. Condorelli et al. have shown that the number of spermatozoa with high mitochondrial membrane potential (MMP) has been increased by the use of Myo-Ins, reducing on the other end those ones with low MMP in patients with OAT [18].

In this study, it was shown that Myo-Ins is able to increase significantly the total and progressive sperm motility in fresh samples before and after capacitation. As the cryopreservation of reproductive technologies is an important strategy for fertility and functional sperm preservation, especially when cycles of chemotherapy and radiation therapy or genetic predisposition can reduce the individuals' reproductive potential, the evaluation of frozen samples was carried out. The freezing process is quite stressful for all types of cells, but spermatozoa undergo little or no structure change during this event, causing the small cell volume and the compact cellular organization of the sperm head. Despite this, after thawing, motility generally is reduced by 30–50%, diminishing also sperm quality and fertilization rate. However, sperm quality is not impaired directly by the freezing technique, but mainly by the biochemical characteristics of the sample itself at baseline [31].

The effect of Myo-Ins was also compared on thawed samples with pentoxifylline, a methylxanthine derivative, nonspecific inhibitor of phosphodiesterase with stimulatory effect on motility due to the increase in cAMP. In literature, the beneficial effects of pentoxifylline on the motility of fresh [32, 33] and cryopreserved sperm [34] are reported. However, few studies have also revealed conflicting results due to a toxic effect on sperm [35] and a possible embryo toxicity in rats [36]. For these reasons, actually, it is not used more to increase motility of sperm sample for IVF techniques but only to detect the vital spermatozoa in samples with total lack of motility (e.g., OAT or testicular spermatozoa) when performing techniques such as intracytoplasmic sperm injection (ICSI).

Treatment with Myo-Ins on thawed samples was more efficacious than pentoxifylline showing a significant difference in improving progressivity. So, it would be interesting to investigate the efficacy of Myo-Ins in the temporary restoring of motility in immotile spermatozoa, in order to evaluate its possible use as a replacement of pentoxifylline, since it has not shown toxicity and has proved to be well tolerated.

Competing Interests

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

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Research Article

Myo-Inositol Safety in Pregnancy: From Preimplantation Development to Newborn Animals

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Myo-inositol (myo-Ins) has a physiological role in mammalian gametogenesis and embryonic development and a positive clinical impact on human medically assisted reproduction. We have previously shown that mouse embryo exposure to myo-Ins through preimplantation development *in vitro* increases proliferation activity and blastocyst production, representing an improvement in culture conditions. We have herein investigated biochemical mechanisms elicited by myo-Ins in preimplantation embryos and evaluated myo-Ins effects on postimplantation/postnatal development. To this end naturally fertilized embryos were cultured *in vitro* to blastocyst in the presence or absence of myo-Ins and analyzed for activation of the PKB/Akt pathway, known to modulate proliferation/survival cellular processes. In parallel, blastocyst-stage embryos were transferred into pseudopregnant females and allowed to develop to term and until weaning. Results obtained provide evidence that myo-Ins induces cellular pathways involving Akt and show that (a) exposure of preimplantation embryos to myo-Ins increases the number of blastocysts available for uterine transfer and of delivered animals and (b) the developmental patterns of mice obtained from embryos cultured in the presence or absence of myo-Ins, up to three weeks of age, overlap. These data further identify myo-Ins as a possibly important supplement for human preimplantation embryo culture in assisted reproduction technology.

1. Myo-Inositol

Myo-inositol (myo-Ins) is the major component of a family of nine hexahydroxycyclohexane inositol (Ins) stereoisomers broadly distributed in eukaryotic tissues and cells, where they are involved in basic biological functions [1].

Ins is incorporated into cell membrane phosphoinositides or phosphatidyl-glycerides as phosphatidyl-inositol (PI), which is phosphorylated by a set of specific phosphoinositide-3-kinases (PI3K) to phosphatidyl-inositol phosphates. Phosphatidyl-inositol trisphosphate (PIP3) can be further metabolized by phospholipase C (PLC) to inositol trisphosphate (Ins–1,4,5P3, InsP3), which acts as second messenger for various hormones such as FSH, TSH, and insulin [2, 3]. Inositol is the precursor of other membrane components and glycosyl-phosphatidyl-inositol (GPI) protein-anchor molecules (reviewed by [4]), signaling molecules (reviewed by [5, 6]), chromatin-remodeling, and transcription-modulating molecules [7]. It finally acts as a hyperosmotic stress protectant [8].

2. Myo-Inositol in Reproduction, Gametogenesis, and Embryonic Development

Increasing evidence shows that myo-Ins has a physiological role in mammalian gametogenesis and embryonic development and a positive clinical impact on human medically assisted reproduction.

Together with its isomer D-chiro-inositol (D-chiro-Ins), myo-Ins acts as second messenger of insulin [9] and has been widely studied for its involvement in metabolic syndrome (reviewed by [10]) as well as for the treatment of polycystic

ovary syndrome (PCOS) (reviewed by [11, 12]), one of the most common female endocrine disorders [13] strictly associated with insulin resistance [14]. Recent reports strongly suggest that both Ins isomers can be positively associated in the management of PCOS patients enrolled in assisted reproduction procedures [15], with significant encouraging clinical outcomes [16].

While D-chiro-Ins does not appear to have a clear role in the ovary or in the testis, myo-Ins affects gametogenetic and embryogenetic processes at several levels, having a protective, positive role in reproduction and development (reviewed by [17]).

Myo-Ins concentration in the reproductive tracts of male mammals is higher than in blood serum being produced by FSH-responsive Sertoli cells [18, 19]. Studies performed on pathological sperm samples have shown that myo-Ins is crucial in spermatogenesis by positively affecting sperm cell motility [20] and mitochondrial membrane potential [21], an apoptotic marker directly associated with cell viability. Since these parameters are predictors of good embryos quality, the use of myo-Ins in the andrology laboratory is now employed to improve the recovery of sperm cells after swim-up in *in vitro* fertilization (IVF) cycles [20].

As for female gametogenesis, myo-Ins is actively concentrated in mammalian follicular and tubal fluid, being higher than in blood serum [22]. Respective values of myo-Ins concentration in human follicular fluid and serum are 30-to-40 μM [23] and 10-to-20 μM [24]. In addition, a positive correlation exists between follicular myo-Ins content and oocyte quality as well as pregnancy outcome [23].

At the ovarian level, Myo-Ins has different functions in both follicle cells and oocytes. However, although it is known that myo-Ins metabolism is severely deregulated in follicle cells of PCOS patients [25], effects of this molecule on somatic ovarian cells still need to be evaluated. We have hypothesized that in theca and granulosa cells myo-Ins may sustain steroidogenic activity during the ovarian cycle by modulating the dynamics of cytoskeletal structures [26]. Research on this issue is ongoing in our laboratories.

Myo-Ins is transported into mammalian cells, including growing and fully-grown oocytes and early preimplantation embryo blastomeres, by a sodium cotransporter [27] and a sodium independent transporter [28]. In oocytes, activity of myo-Ins–derived InsP3 on the modulation of intracellular Ca^{2+} concentration in response to the action of the hormones LH and FSH [29, 30] is well known. It acts via cell-specific receptors (InsP3-R1) [31] and appears to play a key role in meiotic maturation [32].

Chiu et al. [33] cultured preovulatory oocytes from outbred mice in minimal essential medium supplemented with myo-Ins and observed increased rates of maturation, IVF, and development to the two-cell stage with respect to oocytes cultured in medium alone, suggesting that higher availability of myo-inositol increases both meiotic and activation efficiencies of the oocyte. After transfer to foster mothers, the implantation rate and postimplantation viability of these embryos were also increased in the myo-Ins treated groups [33].

During mammalian preimplantation development, activity of myo-Ins transporters ensures a robust uptake, which progressively increases from the one-cell to the blastocyst stage [34, 35], suggesting a parallel increase in cellular requirement of the molecule. It has been shown that, in preimplantation embryo blastomeres, myo-Ins is rapidly incorporated into phosphoinositides [34], leading to raised intracellular InsP3 levels. This led several groups, including ours, to investigate whether myo-Ins could also have a positive action during preimplantation development in both the laboratory mouse and farming species.

Myo-Ins supplementation of culture media was found to improve rabbit and bovine blastocyst formation and expansion [35, 36]. Culture of rabbit embryos at the morula stage in medium containing myo-Ins at the optimal concentration of 75 μM resulted in blastocysts expansion with a fourfold increase in diameter when compared to that provided by standard culture conditions [35]. Similar observations were obtained on bovine zygotes matured and fertilized *in vitro* [36], after culture in *synthetic oviduct fluid* medium [37] in the presence or absence of 2.77 mM myo-Ins. As a result, blastocyst rate was higher among embryos developed in the presence of myo-Ins [36]. To evaluate postimplantation effects of this treatment, ten blastocysts grown in the presence of myo-Ins were transferred to foster cows and developed to term, producing five healthy animals [36].

With a clear interest in human assisted reproduction technology (ART), positive data on myo-Ins in mammalian preimplantation development prompted us to hypothesize that inclusion of myo-Ins in embryo culture media would result in increased numbers of high quality embryos produced by IVF/intracytoplasmic sperm injection (ICSI). We tested this hypothesis in a previous work [38], using the mouse embryo model [39, 40], by investigating the effects of myo-Ins supplementation of sequential human embryo culture media, starting 30 minutes after fertilization (p.f.) and for the whole length of preimplantation development (day 4 p.f.). In that study, we cultured embryos obtained by ICSI from gametes of inbred C57BL/6N mice, in which ICSI results in suboptimal rates of oocyte survival and blastocyst development, when compared to hybrid mice [41]. After fertilization in unmodified fertilization medium, embryos were cultured in cleavage medium in the presence or absence of 10 mM myo-Ins (myo-Ins+ and myo-Ins−, resp.) and monitored daily for developmental progression. At all-time intervals monitored, (p.f.), myo-Ins+ embryos displayed a faster cleavage rate, with a higher percentage of embryos at the most advanced stage. In these embryos, early differentiative events such as compaction and blastulation occurred at the proper developmental stage, excluding apparent toxic effects of myo-Ins. Although results scored on day 4 have already been published [38], those recorded at intermediate times have not been presented elsewhere and are reported in Figures 1 and 2, together with embryo morphology observed on day 4.

Embryos cultured in the presence of myo-Ins were developmentally advanced with respect to control embryos, being mostly represented by expanded blastocysts with a higher number of blastomeres, as shown by Hoechst 33343 nuclear

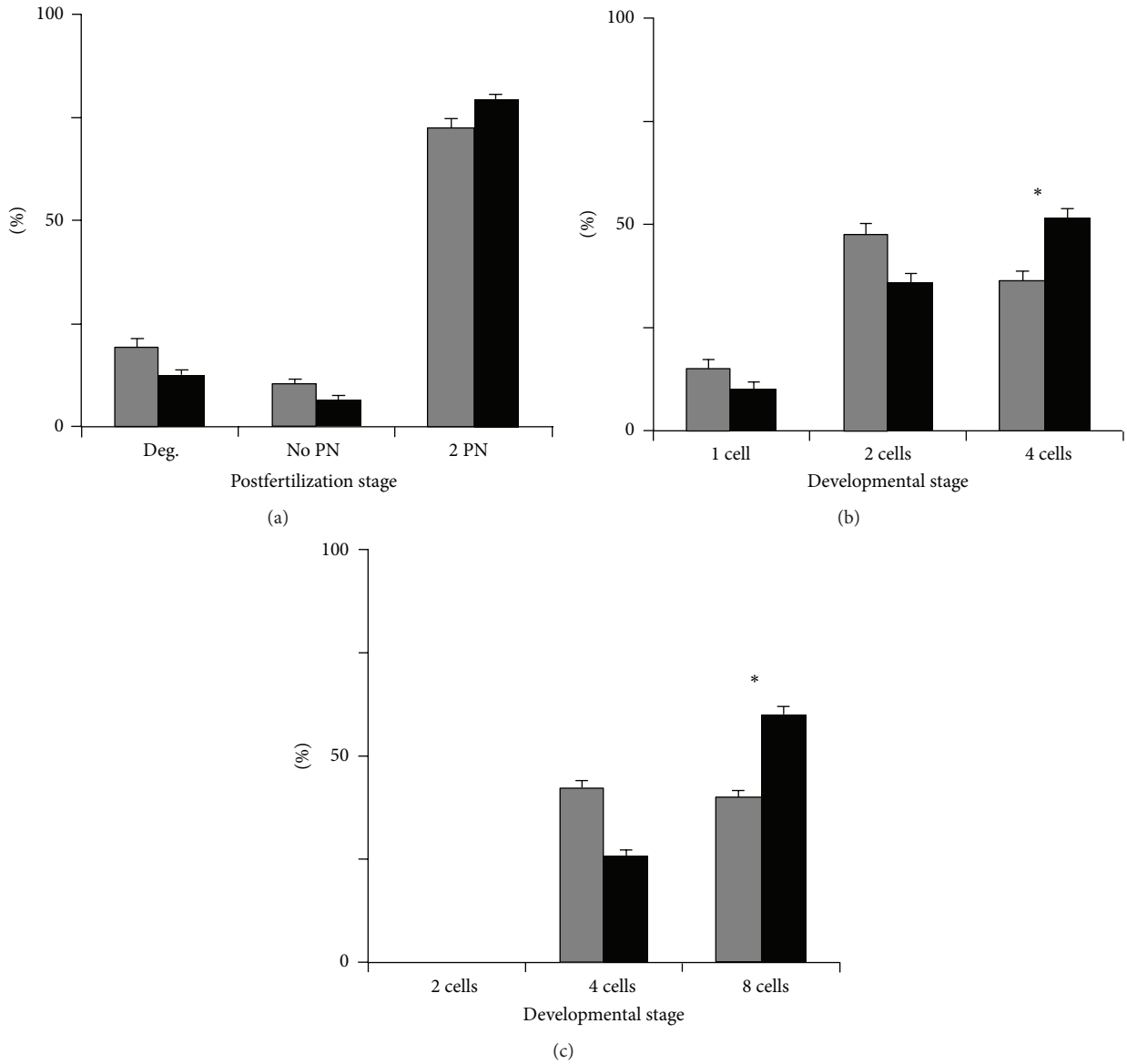

Figure 1: Effect of myo-inositol medium supplementation on pronuclear formation and embryo development during the first two days of *in vitro* culture. Completion of early developmental steps by zygotes cultured in the absence (grey bars) or the presence (solid bars) of 10 mM myo-inositol. Zygotes were scored 6 hours (a) and embryos at 24–26 hours (b) and 48–50 hours (c) p.f. Bars represent the mean ± SEM of 7 independent experiments of the fraction of embryos at each indicated stage. (a) N, myo-Ins– embryos (grey bars), 121; myo-Ins+ embryos (solid bars), 123. ((b), (c)) N, myo-Ins– embryos (grey bars), 97; myo-Ins+ embryos (solid bars), 106. Asterisks indicate difference between treatments calculated by χ^2 test with Yates correction for continuity: (b) $P < 0.05$; (c) $P < 0.01$.

staining [38]. We concluded that myo-Ins supplementation represents an improvement of culture conditions reducing the developmental gap typically observed between embryos obtained and cultured *in vitro* and those developed *in vivo,* further supporting its possible use for human embryo preimplantation culture.

One of the issues left uncovered by these experiments concerns the nature of biochemical pathways induced by exposure to myo-Ins in preimplantation of embryo blastomeres. Existing information suggests that possible pathway(s) elicited by myo-Ins in mouse embryo blastomeres and responsible for a reduced length of cell cycles and a more efficient proliferation activity are initiated via rapid incorporation into phosphoinositides [34], namely, PIP3, enzymatically fostered by activity of PI3K. In turn, PIP3 is converted by PLC into diacylglycerol and InsP3, which, by mobilizing Ca^{2+} stores, has already been recognized to speed up several cellular processes, including proliferation [42, 43]. PI3K is also responsible for the activation of the PKB/Akt pathway [44], which is known to promote proliferation of mouse embryo blastomeres [45]. As myo-Ins has been shown to increase Akt phosphorylation and hence its activity in mouse skeletal muscle cells [46], we hypothesized that a critical step in enhancing embryo preimplantation

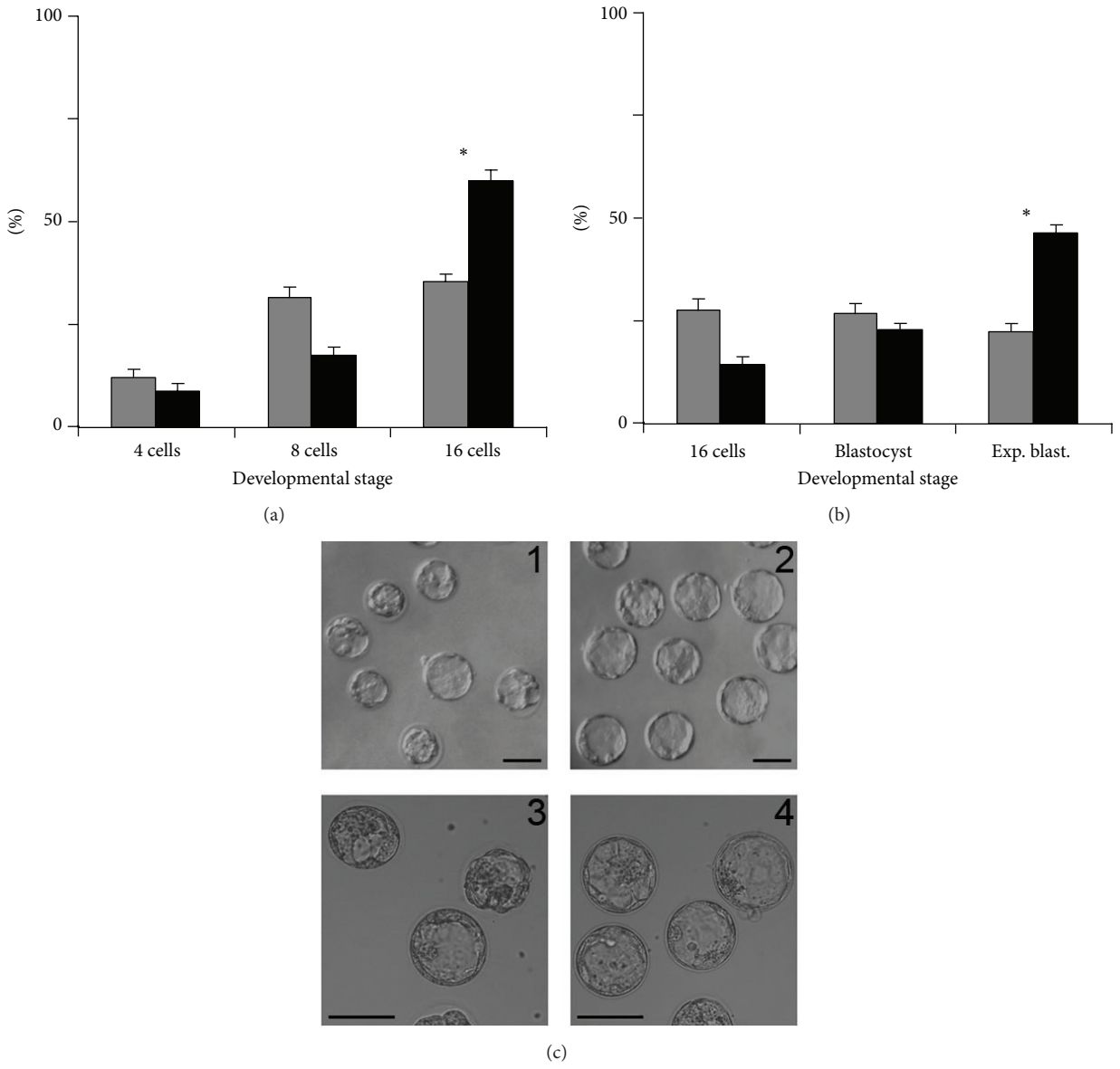

Figure 2: Effect of myo-inositol medium supplementation on embryo development during the third and fourth days of *in vitro* culture. Completion of mid-to-late preimplantation development by embryos cultured in the absence (grey bars) or the presence (solid bars) of 10 mM myo-inositol. Embryos were scored 72–74 hours (a) and 96–98 hours (b) p.f. Bars represent the mean ± SEM of 7 independent experiments of the fraction of embryos at each indicated stage. ((a), (b)) N, myo-Ins− embryos (grey bars), 97; myo-Ins+ embryos (solid bars), 106. Exp. blast.: expanded blastocyst. Asterisks indicate difference between treatments calculated by χ^2 test with Yates correction for continuity: (a) $P < 0.05$; (b) $P < 0.05$. (c) Representative images of expanded and nonexpanded blastocysts observed at day 4 among myo-Ins− (1, 3) and myo-Ins+ (2, 4) embryos; magnification: 40x (1, 2); 80x (3, 4). Black bars, 100 μm. Panel (b) is modified from [38].

development may be represented by Akt activation induced by myo-Ins supplementation [47]. We here provide the first evidence, obtained by immunofluorescence analysis, that myo-Ins supplementation of culture media increases serine phosphorylation of PKB/Akt in mouse preimplantation embryos.

A second open issue in current research concerns the safety of preimplantation embryo exposure to myo-Ins for postimplantation and postnatal development.

Besides the reported observations on bovine embryos [36], it could be argued that preimplantation embryos, including human embryos, were once routinely cultured in undefined media supplemented with commercial BSA preparations or batches of bovine or human sera, which contain variable amounts of myo-inositol, without reporting any apparent negative effect.

In addition, myo-Ins appears important for normal development (reviewed by [17]). Its concentration in foetal human serum is severalfold higher than in adults, lowering only around birth [48], and its administration during pregnancy has positive effects on pathological conditions in both humans [49, 50] and rodent species [51–54].

All data hitherto reported suggest that preimplantation embryo exposure to myo-Ins would have no detrimental effects on further development. To obtain more direct information on this issue, we transferred blastocysts produced *in vitro* in the presence of myo-Ins into recipient foster mothers and allowed their development to term. By this approach, we obtained healthy offspring that appeared normal in the sex ratio and, at least until weaning, somatometrically. These experiments provide additional data on myo-Ins effects on mammalian preimplantation embryos and strongly suggest that it can be considered safe for embryo development to term.

3. Materials and Methods

3.1. Animals. Animals (Charles River Italia, Calco, VA, Italy) were housed in a temperature-controlled facility (22 ± 1°C) on a 12/12 h light/dark cycle, inside standard cages with unlimited access to food and water. Forty-to-60-day-old C57BL/6N mice were used as donors of oocytes and male studs. Forty-to-60-day-old CD/1 mice were used as foster mothers and vasectomized male studs. All experimental procedures were conducted in accordance with the official European guidelines for the care and use of laboratory animals (86/609/EEC). Experimental protocols and related procedures were approved by the Italian Ministry of Public Health.

3.2. Culture Media. In order to model human preimplantation embryo culture, all procedures were performed in commercial Quinn's Advantage media and supplements (BioCare Europe, Napoli, Italia), which do not contain myo-Ins, as described [38].

3.3. Animal Treatment and Zygote Collection. Female mice were hormonally induced by intraperitoneal injections of 5 IU pregnant mare serum gonadotropin (NHPP, Torrence CA, USA) and, 46–48 h later, of 5 IU human chorionic gonadotropin (Corulon, Intervet Italia, Aprilia, LT, Italy). After mating with male mice ON, females carrying a vaginal plug were sacrificed by cervical dislocation and cumulus-oocyte complexes were collected from the oviducts, freed from cumulus cells by treatment with 0.1% hyaluronidase in Hepes buffered Quinn's Protein Plus Advantage HTF-Medium containing 5% human serum albumin, rinsed in HTF-Medium, and incubated at 37°C in Quinn's Protein Plus Cleavage Medium (C-Medium) under a humidified atmosphere of 5% CO_2 in air.

3.4. Embryo Culture. One-cell embryos were positively scored by the presence of two pronuclei and then divided into two groups. One group was further cultured in 14 μL/mL myo-inositol (10 mM, Andrositol® LAB Lo.Li. Pharma, Italy) in C-Medium and the other one in 14 μL/mL phosphate buffered saline in C-Medium. Embryo culture was performed in 500 μL medium inside Falcon 4 well IVF plates without medium replacement, as described [38]. Developing embryos were scored daily for morphology and progression through cleavage stages.

3.5. Detection of Phosphorylated PKB/Akt. Preimplantation embryos at the morula or blastocyst stage were washed in 0.1 M phosphate buffered saline, pH 7.4 (PBS), containing 0.3% bovine serum albumin (BSA) (PBS-BSA), and were then fixed in 4% paraformaldehyde in PBS for 30 min at room temperature (RT). After three washes in PBS-BSA, embryos were permeabilized in PBS containing 0.25% Tween-20 (Sigma; St. Louis, MO) for 15 min at RT and washed three times in PBS-BSA. Permeabilized embryos were processed for immunostaining by an overnight incubation at 4°C in the presence of the primary antibody, rabbit monoclonal anti-phospho-Akt (Ser473, #4080), anti-phospho-Akt (Thr308, #4056) or anti-Akt (total, #9272) antibody (Cell Signaling Technology, Beverly, MA, USA) (1 : 75), followed by washing in PBS-BSA and 1 hr final incubation with the secondary, and Alexa Flour-488 conjugated secondary antibodies (Invitrogen), diluted 1 : 200 in PBS-BSA for 1 h at RT. After washing with PBS-BSA, blastomere nuclei were counterstained with 1 mg/mL Hoechst 33343 for 10 min at RT. Finally, embryos were washed three times for 10 min each in PBS-BSA and mounted in PBS and glycerol (1 : 1 v/v) using coverslips. Fluorescent signals were detected using a Zeiss AxioPlan fluorescence microscope (Carl Zeiss, Oberkochen, Germany) at 400x magnification. For semiquantitative analysis of fluorescence, embryos at various developmental stages were immunostained after pooling in the same drops. Fluorescence emission was collected under similar excitation conditions and then quantitatively analyzed by using the ImageJ software (ImageJ 1.47v, Wayne Rasband, National Institutes of Health, USA. http://imagej.nih.gov/ij/).

3.6. Embryo Transfer and Development to Term. In ten replicate experiments, embryos developed to the blastocyst stage after 4 days of culture in myo-Ins+ C-Medium or myo-Ins− C-Medium were transferred to the uteri of pseudopregnant foster mothers mated 2.5 days earlier with vasectomized males and carried to term [55]. On the day of delivery, newborn animals were weighed, checked for gross abnormalities, and left to be nursed by their moms until weaning. Preweaning morphological analyses included body growth at one week, fur appearance, and eye opening. Mice were finally weighed and sexed at weaning (three weeks of age).

3.7. Chemicals. Where not stated otherwise, chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

3.8. Data Analysis. Data were analyzed by using Student's t-test or χ^2 tests with Yates continuity correction. Statistical analyses were performed using R: A language and environment for statistical computing (R development core team, R foundation for statistical computing, ISBN 3-900051-07-0, 2008, Vienna, Austria. https://www.r-project.org/).

4. Results

4.1. Analysis of Myo-Ins−Dependent Akt Phosphorylation. We have determined presence and phosphorylation of Akt in late preimplantation stage embryos cultured in the presence

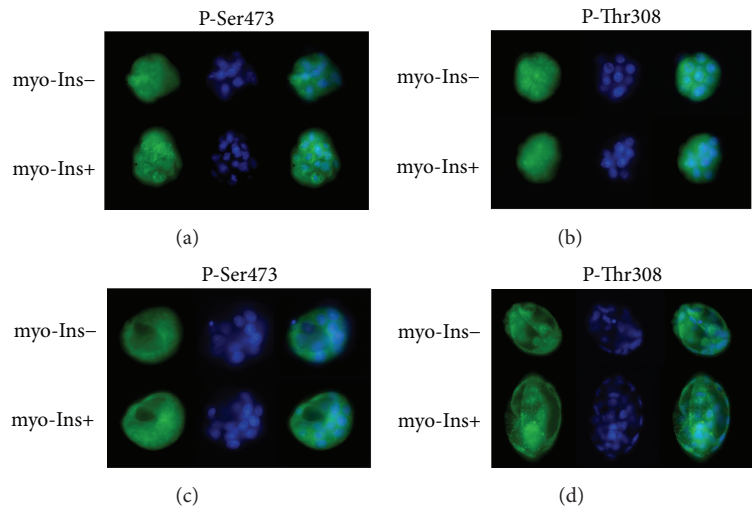

Figure 3: Localization of phosphorylated Akt in preimplantation embryos at the morula and blastocyst stage, after continuous culture in the presence or absence of myo-Ins. ((a), (b)) morula stage embryos; ((c), (d)) blastocyst stage embryos. In each panel, from left to right: FITC, Hoechst 33343, merge. The same pattern was consistently observed in all embryos analyzed; panels show representative embryos. Statistical analysis on these embryos, reported in the text, was performed on at least three embryos at the morula and blastocyst stages in three independent experiments.

Table 1: Effect of myo-Ins on Akt phosphorylation of late preimplantation mouse embryos.

	Morulae		Blastocysts	
	Ser473	Thr308	Ser473	Thr308
Myo-Ins+	65.41 ± 5.01	47.77 ± 4.37	49.33 ± 6.16	32.15 ± 1.56
Myo-Ins–	59.80 ± 2.55	45.87 ± 2.23	38.32 ± 2.54[a]	33.09 ± 2.03

Data represent arbitrary fluorescence units (mean ± SD) of embryos at the stages indicated, pooled from three replicate experiments.
[a] $P = 0.02$, calculated by Student's t-test.

Table 2: Mouse development after IVF and exposure of preimplantation embryos to myo-Ins.

	Number of fertilized oocytes	Number of transferred blastocysts (%)	Number of delivered animals (%)
Myo-Ins+	154	105 (68.2)	59 (38.3)
Myo-Ins–	147	76 (51.7)[a]	33 (22.5)[a]

Data were pooled from ten replicate experiments.
Superscripts indicate differences between myo-Ins+ and myo-Ins– embryos ($P < 0.05$) calculated by χ^2 test; differences are referred to fertilized oocytes.

or absence of myo-Ins by immunofluorescence analysis. This approach revealed the presence of serine 473- and threonine 308-phosphorylated Akt in both morula and blastocyst embryos (Figure 3). Phosphorylated Akt was localized prominently in blastomere cytosols but a limited nuclear localization was also observed. The relative content of phosphorylated Akt at the same stages was also measured by quantification of immunofluorescence data (Table 1). The level of serine 473-phosphorylated Akt did not appear to be modified in embryos cultured in the presence of myo-Ins at the morula stage ($t4 = 1.75$, $P = 0.15$) (Figure 3(a)) but it was increased at the blastocyst stage ($t4 = 3.61$, $P = 0.02$) (Figure 3(c)). On the contrary, the level of threonine 308-phosphorylated Akt did not vary significantly depending on the presence or absence of myo-Ins (morulae, $t4 = 0.670$, $P = 0.54$, Figure 3(b); blastocysts, $t4 = 0.640$, $P = 0.56$, Figure 3(d)). Amounts of total Akt did not vary under different conditions (not shown).

4.2. Effects of Preimplantation Embryo Exposure to Myo-Ins on Development to Term. In ten replicate experiments, all embryos that had developed *in vitro* to the expanded or nonexpanded blastocyst stage in the presence or absence of myo-Ins were transferred to foster mothers and allowed to develop through birth and until weaning. Of 105 myo-Ins+ and 76 myo-Ins– blastocysts transferred, 59 (56.2%) and 33 (43.4%), respectively, were delivered ($\chi^2 = 2.876$, $P = 0.09$). Although this difference was not significant, when we compared numbers of delivered animals with numbers of fertilized oocytes cultured under the two conditions, 154 myo-Ins+ one-cell embryos and 147 myo-Ins– one-cell embryos, we obtained a significant improvement in the overall efficiency of the treatment ($\chi^2 = 8.91$, $P = 0.003$). A similar difference was observed by comparing numbers of fertilized oocytes with numbers of transferred embryos ($\chi^2 = 8.52$, $P = 0.003$). Overall results are shown in Table 2.

Body weights at birth were 1.42 ± 0.01 g for myo-Ins+ and 1.41 ± 0.02 g for myo-Ins– ($t90 = 0.645$, $P = 0.52$). During the first week, six and four pups were found dead in the myo-Ins+ and myo-Ins– groups, respectively. Somatometric development appeared similar in mice of both groups, with appropriate acquisition of body fur and eye opening. Weights at one week of age were also similar (myo-Ins+, 2.06 ± 0.16 g, myo-Ins–, 2.08 ± 0.19 g; $t80 = 0.286$, $P = 0.78$). Sex distributions in the two conditions were both casual

(myo-Ins+, 27 males, 26 females, myo-Ins−, 14 males, 15 females). Finally, weights at three weeks of age were similar for sexes and embryo culture conditions (males: myo-Ins+, 10.20±0.21 g; myo-Ins−, 10.29±0.28 g, $t39 = 0.790$, $P = 0.43$; females: myo-Ins+, 9.90 ± 0.19 g; myo-Ins−, 9.75 ± 0.40 g, $t39 = 1.214$, $P = 0.23$).

5. Discussion

Akt represents a major downstream effector of growth factors, cytokines, and adhesion receptors capable of promoting cell survival and proliferation [56, 57] and is regulated, among others, by PIP3, the second messenger product of PI3K, via recruitment of PDK1. PDK1 induces direct phosphorylation at threonine 308 [58] and appears to be also implicated in the negative regulation of phosphorylation at serine 473, by direct binding to this hydrophobic site. However, PDK1 can be displaced by an appropriate signal [59, 60], including activation of integrin Like Kinase (ILK) [44], allowing Akt autophosphorylation or phosphorylation by other kinases at serine 473 [58]. This pathway makes Akt phosphorylation of serine 473 inducible by upstream signals; on the contrary, phosphorylation of threonine 308 appears to have a constitutive nature [56].

We have previously shown that, during the first two stages of embryo development, Akt phosphorylation is not prevented by inhibition of either PI3K or PDK1 and concluded that the phosphorylation state and the intracellular distribution of Akt in two-cell embryos are independent of the activity of both kinases. This suggested that Akt is inherited from the oocyte in its phosphorylated/dephosphorylated form at both serine 473 and threonine 308 [45].

We now show by quantitative immunofluorescence analysis that serine 473 phosphorylation of Akt can be increased in late preimplantation embryos by the presence of myo-Ins in the culture medium. It thus appears that, after the initial stages of development, new phosphorylation of Akt may occur in mid-to-late preimplantation stages depending on availability of myo-Ins. In agreement with previous observations that development of mouse preimplantation embryos requires PI3K activity from the 8/16-cell stage [45], it would be reasonable to hypothesize that this finding can be ascribed to the increased production of phosphoinositides [34] and consequent increased production of PIP3 by PI3K. An increase in phosphorylation of Akt may be responsible for the faster developmental rate of embryos cultured in the presence of myo-Ins.

The lack of inducibility of threonine phosphorylation of Akt by myo-Ins is also in agreement with previous observations [56].

It is puzzling that myo-Ins exerts opposite effects on both PI3K and Akt activities when added to cancer cell cultures [61], in which it has been found to reduce PI3K levels as well as Akt activity by inhibiting its phosphorylation. To this respect, it is therefore tempting to speculate that such effect could be considered context-dependent and that some other still unknown factors are likely to participate in modulating the PI3K/Akt pathway. In order to obtain more specific information on this pathway, we are currently analyzing the effects of myo-Ins both on development and on Akt phosphorylation in preimplantation embryo in the presence of inhibitors of PI3K and PDK1 [45]. Further experiments will address the involvement on proliferative activity of preimplantation embryos blastomeres of pro- and anti-apoptotic factors of the Bcl-2 family [62].

Data here produced represent a first assessment of the effect of preimplantation embryo exposure to myo-Ins on mouse development to term.

So far, information on this issue in mammals is limited to one finding obtained on bovine embryos cultured in the presence of 2.77 mM myo-Ins [36]. In that study no comparison was made between embryos cultured in the two conditions, but blastocysts that had developed after preimplantation exposure to myo-Ins were transferred producing healthy animals.

Present results obtained in the mouse show (a) the apparent absence of early toxic effects of myo-Ins, as suggested by normal prenatal and short-term postnatal development, and (b) a significant increase in the overall rate of live births obtained after preimplantation embryo culture in myo-Ins and subsequent transfer into foster mothers.

If the first observation was expected in light of the body of information here reported on the positive effects displayed by myo-Ins on mammalian gametogenesis and development, the second one deserves particular attention. In fact, it supports the possibility that a regular use of myo-Ins as culture supplement provides high efficiency in the production of viable preimplantation embryo *in vitro* both in the mouse and in farming specie with promising outcome for both scientific and economic purposes.

In addition, it strengthens the hypothesis that the use of myo-Ins would have a similar positive role in the culture of *in vitro* produced human embryos, with obvious medical consequences.

To this end, however, additional assessments of myo-Ins effects are necessary at least at three different levels [63]: (a) on the expression of imprinted genes during development; (b) on the acquisition of sensory/motor/behavioral functions during early development; and (c) on long-term consequences on the whole organism.

Disclosure

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Competing Interests

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

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Review Article

How to Achieve High-Quality Oocytes? The Key Role of Myo-Inositol and Melatonin

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Assisted reproductive technologies (ART) have experienced growing interest from infertile patients seeking to become pregnant. The quality of oocytes plays a pivotal role in determining ART outcomes. Although many authors have studied how supplementation therapy may affect this important parameter for both in vivo and in vitro models, data are not yet robust enough to support firm conclusions. Regarding this last point, in this review our objective has been to evaluate the state of the art regarding supplementation with melatonin and myo-inositol in order to improve oocyte quality during ART. On the one hand, the antioxidant effect of melatonin is well known as being useful during ovulation and oocyte incubation, two occasions with a high level of oxidative stress. On the other hand, myo-inositol is important in cellular structure and in cellular signaling pathways. Our analysis suggests that the use of these two molecules may significantly improve the quality of oocytes and the quality of embryos: melatonin seems to raise the fertilization rate, and myo-inositol improves the pregnancy rate, although all published studies do not fully agree with these conclusions. However, previous studies have demonstrated that cotreatment improves these results compared with melatonin alone or myo-inositol alone. We recommend that further studies be performed in order to confirm these positive outcomes in routine ART treatment.

1. Introduction

Female infertility, defined as a failure to achieve a successful pregnancy after 12 months or more of appropriate, timed unprotected intercourse or therapeutic donor insemination [1], has various causes and elements that play a key role including, aging, acute and chronic diseases, pharmacological therapies, behavioral factors, and exposure to environmental, occupational, and infectious agents. Data from 2006 to 2010 from the Center for Disease Control (CDC) National Survey of Family Growth show that, among all married US women aged 15–44 years, 6% (1.5 million women) were infertile and 12% (3.1 million women) had impaired fertility, defined as a condition in which there is physical difficulty in getting pregnant or carrying pregnancy to term [2]. These figures would be even more alarming if the data included statistics regarding couples who tried for one year or more to become pregnant and failed [3].

In recent decades, maternal age has progressively increased: in 1975 only 5% of women who became pregnant were over 30 years old, whereas in 2010 this percentage was 26% [4, 5]. Aging is one of the principal factors related to

female fertility, with a direct correlation between increased maternal age and infertility/subfertility rates, probably due to diminished egg quality, ovulatory dysfunction, and reproductive disorders such as tubal diseases, leiomyoma, and endometriosis [6]. According to recent data, the fertility decline begins at the middle of third decade [7]. Reproductive outcomes depend directly on the quality of oocytes, which develop proper competence according to their genetic and epigenetic programming. The integrity of cytoskeleton, mitochondria function, and distribution play a pivotal role during the spindle formation and consequently modulate chromosomal segregation and maintain genome stability after division [8, 9]. Spindle is composed of bundles of microtubules, which are polar polymers of α- and β-tubulin heterodimers. The most common defect in aging is shortening and disorganization of spindle, and this alteration may predispose the oocyte to aneuploidy or maturation arrest. In addition, mitochondria and endoplasmic reticulum control calcium-mediated intracellular signaling. This is of paramount importance for oocyte activation and modulates calcium/calmodulin-dependent protein kinase II, which is implicated in the transition from metaphase I to anaphase I [8].

Currently many infertile women undergo assisted reproductive technologies (ART) [10], dating from the first baby born after in vitro fertilization (IVF) and embryo transfer (ET) [11]. Thereafter, women affected by primary ovarian failure became pregnant with egg donation [12], a common procedure today [13]. Other techniques have been developed, such as gamete intrafallopian transfer (GIFT) and zygote intrafallopian transfer (ZIFT), although these are not universally applicable, for example, for women with tubal occlusion or in the case of male infertility [10]. ART has continued to grow with improvements in ultrasonography, including ultrasound-guided transvaginal follicular aspiration [14] with a consequent reduction in women's health risks and expenses. Intracytoplasmic sperm injection (ICSI) [15] was a step forward which improved outcome in the case of male infertility. Today IVF represents 99.5% of ART [13]. With growing interest in IVF as an answer to infertility, the interest of the scientific community is focused on supplementation, which can improve the outcome of these techniques.

2. Rationale for the Use of Melatonin

In recent years, attention has been focused on the negative impact of oxidative stress due to an excess of free radicals on oocyte quality during the IVF cycle. Free radicals are represented by reactive oxygen species (ROS), the most important class generated in living systems [16] and reactive nitrogen species (RNS). Free radicals play a dual role with both deleterious and beneficial effects. Physiologically free radicals maintain a balance in cells, called "redox balance" according to both its production and antioxidant cellular capacity. These are represented by both enzymatic antioxidant defenses, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and nonenzymatic antioxidant defenses, like ascorbic acid, α-tocopherol, glutathione (GSH), carotenoids, flavonoids [17], and other molecules such as melatonin.

The overproduction of ROS results in oxidative stress, which can be an important mediator of damage to cell structures as lipids, membranes, proteins, and DNA [18]. On the other hand, free radicals are involved in cellular signaling pathways, in the induction of mitosis, and in defense against *noxa* as infectious agents. One physiological function in which ROS plays an fundamental role is reproductive physiology, including follicular development, oocyte maturation, ovulation, corpus luteum function, and follicular atresia [19]. Several authors compared ovulation to an inflammatory reaction with the production of cytokines and free radicals [19, 20]. The follicular fluid (FF) obtained from antrum of Graafian follicles has a higher melatonin concentration than a plasma sample collected simultaneously [21]. This is probably related to the necessity to protect the oocyte from free radicals formed during oocyte maturation and to preserve oocyte quality [22]. As support, one study indicated that reduced antioxidant enzyme level was reported in the FF of women with unexplained infertility [23].

In 1993 it was discovered that melatonin is a potent endogenous scavenger of free radicals [24]. Before then melatonin was known as a hormone, produced by the pineal gland, with the ability to modulate circadian and reproductive physiology in photoperiod-dependent, seasonally breeding mammals [25]. Today it is known that melatonin links its receptors in the suprachiasmatic nuclei of the brain and in pars tuberalis in the pituitary gland to modulate the reproductive function. The presence of melatonin receptors is found in many other tissues of the reproductive system (epididymis, vas deferens, prostate, ovary, and mammary gland) [26] and other tissues including the skin, gastrointestinal tract, liver, kidney, spleen, and immune system cells. Enzymes involved in melatonin synthesis have been found in most of these tissues. These findings may relate to a hypothesis of multiple functions performed by melatonin (antioxidant, anti-inflammatory properties, genomic stabilizing effects, and modulator of mitochondrial homeostasis) [27].

Melatonin acts directly as a scavenging free radical [28] or in binding with its receptors [29] to prevent damage in cells and tissues. The antioxidant melatonin mechanism is different from other antioxidants because, after reacting with free radicals in a "scavenging cascade reaction," it generates several stable antioxidant end-products. It does not promote oxidation under any circumstances as this would terminate its properties as an antioxidant. In addition, melatonin synergizes with other nonenzymatic antioxidants [30] and also stimulates antioxidant enzyme synthesis by binding its membrane receptors to cytosolic or nuclear-binding sites [31].

Tamura et al. [32] measured melatonin concentration in FF in humans during oocyte retrieval for IVF-ET and correlated it with levels of 8-hydroxy-2-deoxyguanosine (8-OHdG), which is a product of free radical damage. They found an inverse correlation between two molecules, suggesting that 8-OHdG is an index of degeneration of oocytes. Furthermore, the inverse relation between melatonin and 8-OHdG may confirm the protective role of melatonin against oxidative stress [32]. In line with this last finding, a recent

study [33] showed that mature follicles have a higher level of melatonin compared to immature follicles and that melatonin is secreted from luteinizing granulosa cells (GC). The study measured mRNA of enzymes involved in melatonin synthesis and found increased levels of mRNA in GC, which were able to be synthesized by this hormone. Based on these data, it is possible to speculate that melatonin may be released from luteinizing GC during late folliculogenesis and is involved in oocyte maturation.

During IVF, oocyte and embryos may be exposed to high levels of superoxide free radicals started during the stimulation protocol, in which oocyte manipulation alters the level of endogenous oxygen scavengers [34]. FF rich in antioxidants is absent in oocyte medium cultures including melatonin, which protects oocyte from oxidative stress. Furthermore the ROS level is higher during IVF due to a high oxygen concentration level in incubators and handing throughout the IVF process [35]. With the increase in "oxidative stress" during IVF, melatonin, with its scavenger properties, finds its proper use.

3. Use of Melatonin in ART

It was observed that adding melatonin in mouse oocyte cultures resulted in 54% of fertilized oocytes that reached the blastocyst stage compared to 29% without melatonin [36]. Other authors studied porcine oocytes cultures and found that adding melatonin to oocyte culture media produced a significantly lower level of ROS and a greater proportion of mature oocytes (MII) [37]. Recent evidence suggests that a concentration of melatonin in oocyte culture should be in a narrow range between 10^{-6} and 10^{-9} nM to achieve the best results.

Some authors found that in humans supplementation with a low dose of melatonin improved oocyte nuclear maturation, and conversely decreased nuclear maturation was observed with a high dose [38]. A range from 10^{-5} to 10^{-9} nM improved oocyte maturation when added at in vitro oocyte maturation (IVM), and it augmented the implantation rate during IVF-ET, increasing the pregnancy rate in women with PCOS, although in some cases the increase was not significant [33].

Tamura et al. [32], in addition, showed that oral administration of melatonin raised the concentration of melatonin in FF, and this higher concentration was inversely related to cellular oxidative stress measured by the 8-OHdG level. In particular, they studied patients who had undergone unsuccessful IVF, dividing patients into two groups: the first treated with orally administered melatonin (3 mg/day) and the second without therapy. Considering their data analysis, they found that the first group had a higher fertilization rate than second group.

Other studies show that melatonin significantly improved morphologically MII during IVF compared to patients not treated. In contrast to Tamura et al.'s data [32], these studies did not find an increased fertilization rate, although the number of class 1 embryos was higher in the treated group [39, 40], and pregnancy rates were higher in the treated group although the difference did not reach statistical significance [39].

Other authors showed that the fertilization rate improved with melatonin supplementation both in those with a lower fertilization rate during a previous IVF cycle and in those who underwent an ICSI procedure [41]. For a complete summary of the studies reported in this chapter, refer to Table 1.

4. Rationale for the Use of Myo-Inositol

Inositols are multifaceted molecules that play a fundamental role in many cellular functions. Myo-inositol (MI) and D-chiro-inositol (DCI) are two stereoisomers of a six-carbon cyclitol ring, and MI is a precursor of DCI and is most widely distributed in nature. Inositol is assumed to be an essential B complex vitamin with its main source being a normal diet, although it can be synthetized in the human. Inositols may be present in cells in different ways: incorporated in membrane phospholipids as phosphoinositides [42] and in the form of inositol phosphoglycans (IPG) located in the cytoplasmic membrane, which are involved in insulin transduction signaling as secondary messengers [43]. The role of phosphoinositides has long been known. They are fundamental elements of the cellular membrane, where inositol combines with cytidine diphosphate diacylglycerol to form phosphatidylinositol (PtdIns), a fundamental constituent of cell membranes. Inositol is involved in the phosphoinositides signaling pathway and plays a major role in numerous cellular functions [42]: phosphatidylinositol 4P (PtlIns-4P) and phosphatidylinositol 4,5 bisphosphate (PtdIns 4,5P) are the most studied. Two important second intracellular messengers, diacylglycerol (DAG) and inositol-triphosphate (Ins 1,4,5-P), are produced from PtdIns 4,5P. Ins 1,4,5-P binds Ca2+ channels in the rough endoplasmic reticulum stimulating intracellular Ca2+ release and, subsequently, numerous related intracellular effects. The increase in intracellular Ca2+ levels plays an important role in oocyte maturation, fertilization, and embryo development [44–46]. Intracellular calcium-release mechanisms are modulated during the oocyte maturation process, particularly during the final stages of oocyte maturation. A major sensitivity to calcium release was observed in the final stage [47].

For all these reason, MI is involved in phosphoinositides pathway signaling and plays a key role in oocyte fertilization: many studies describe the relationship of its concentration level in FF, oocyte quality, and estrogen level in FF both in *in vitro* mouse models and in humans during IVF procedures [48, 49].

PtdIns 4,5P is involved in the development of cytoskeleton (CSK) and is involved in the binding between actin and the cellular membrane [42], suggesting a protective function for nuclear chromatin [50]. Another member of PtdIns molecules, PtdIns 3,4,5P, is involved in nuclear modulation of transcriptosome [51]. Some authors speculate that the depletion of inositol may desensitize the PtdIns pathway by slowing down resynthesis of a precursor [52].

Another important aspect of inositols is their role as insulin-sensitizers. In the form of IPG, they are able to

Table 1: Effects of melatonin supplementation therapy (adding in oocytes culture or orally administered to patients) during ART.

Authors	Species	Melatonin in vitro/in vivo	Patients number (tot)	Intervention/control number	Technique	Significant outcomes ($p < 0.05$)
Lord et al. 2013 [36]	Mouse	Adding M in oocyte culture medium				(i) Increased number of oocytes that reached blastocyst stage
Kang et al. 2009 [37]	Porcine	Adding M in oocyte culture medium				(i) Increased mature oocyte
Tamura et al. 2008 [32]	Human	Orally administered M (3 mg/day)	115	56 versus 56 nontreated 1° cycle versus treated 2° cycle	IVF-ET	(i) Improved fertilization rate
		M (3 mg/day) versus Vit. E (600 mg/day) versus M + Vit. E (3 mg + 600 mg/day)	18	59 versus 59 nontreated 1° cycle versus nontreated 2° cycle		(i) Reduced 8-OHdG in FF (ii) Increased in FF in treated patient.
Kim et al. 2013 [33]	Human	Adding M in oocyte culture medium	111	62/49	IVM IVF-ET	(i) Increased mature oocyte (ii) Increased implantation
Batioglu et al. 2012 [39]	Human	Orally administered M (3 mg/day)	85	40/45	IVF-ET	(i) Increased mature oocyte (ii) Increased good quality embryos
Eryilmaz et al. 2011 [40]	Human	Orally administered M (3 mg/day)	60	30/30	IVF-ET	(i) Increased mature oocyte (ii) Increased good quality embryos
Nishihara et al. 2014 [41]	Human	Orally administered M (3 mg/day)	97	97/97 (control of itself)	IVF ICSI	(i) Improved fertilization with ICSI (ii) Improved fertilization in poor responder (iii) Improved good quality embryos

M: melatonin; Vit. E: vitamin E; IVF: in vitro fertilization; ET: embryo transfer; IVM: in vitro maturation; ICSI: intracytoplasmic sperm injection; 8-OHdG: 8-hydroxy-2-deoxyguanosine.

activate insulin signaling pathways. IPG binds insulin with G-protein to create a coupled receptor to act as a secondary messenger, that is, DCI-phosphoglycan, which allows the activation of glycogen synthase both directly and indirectly via phosphatidylinositol 3-kinase (PIK3). It works to activate pyruvate dehydrogenase inside the mitochondria controlling oxidative glucose metabolism [53]. Conversely MI-phosphoglycan, based on IPG, allows the inhibition of cyclic adenosine monophosphate (cAMP) kinase and adenylate cyclase that are a regulatory enzyme in free fatty acid (FFA) metabolism [54].

Previous data analysis [55] showed that the DCI level was 50% lower in muscle and urine samples derived from type II diabetic patients compared with samples from control subjects. Conversely MI did not differ between two groups studied. The imbalance between MI and DCI levels was called "inositol imbalance." The decline in DCI levels and inositol imbalance can be linked to insulin resistance (IR); and DCI levels worsened with the increase of insulin-resistance level [56, 57].

Many studies have shown that in patients with insulin resistance, such as in PCOS patients, using DCI, MI, and MI plus DCI may improve insulin resistance, metabolic parameters, regularity of cycle, and spontaneous ovulation [58–63] in keeping with insulin-sensitizer effects of DCI and the role of MI in oocytes maturation.

However a recent study confirmed that high DCI dosage (higher than 2.4 g/daily) had a negative effect on IVF outcome [64]. These data may explain the DCI ovary paradox theory in which insulin sensitivity remained in IR ovarian cells with a hyperstimulated epimerase function and with an imbalance between lower MI and higher DCI changing the physiologic MI/DCI ratio [65].

5. Use of Myo-Inositol in ART

It has been suggested that MI added to in vitro culture media may increase bovine blastocyst development [66] just as MI improved in vitro maturation in mouse oocytes [67].

Table 2: Effects of myo-inositol supplementation therapy (adding in oocytes culture or orally administered to patients) during ART.

Authors	Species	Myo-inositol in vitro/in vivo	Patients number (tot)	Intervention/control number	Technique	Significant outcomes ($p < 0.05$)
Pesty et al. 1994 [67]	Mouse	Adding MI in oocyte culture medium				(i) Improved oocyte maturation
Chiu et al. 2002 [48]	Human	Adding MI in oocyte culture medium	53	32/21	IVF	(i) Improved good quality oocyte (ii) Increased FF volume
Brusco and Mariani 2013 [68]	Human	Orally administered MI + FA (2 g + 400 mg/day) versus FA (400 mg/day)	149	58/91	IVF-ET ICSI	(i) Improved number of good quality oocytes (ii) Improved number embryos of grade A (iii) Increased clinical pregnancy rate
Ciotta et al. 2011 [69]	Human	Orally administered MI + FA (2 g + 200 mg/twice daily) versus FA (400 mg/day)	34	17/17	IVF-ET ICSI	(i) Increased number oocytes retrieved (ii) Increased numbers embryos transfer (iii) Increased grade S1 embryos (iv) Reduced number of immature oocytes
Unfer et al. 2011 [71]	Human	Orally administered MI (2 g/twice daily) versus DCI (0.6 g/twice daily)	84	43/41	IVF-ET ICSI	(i) Increased numbers mature oocyte (ii) Reduced number of immature oocytes (iii) Increased embryos grade 1
Colazingari et al. 2013 [72]	Human	Orally administered MI + DCI (1.1 g + 27.6 mg/day) versus DCI (0.5 g/day)	100	46/53	IVF-ET	(i) Improved oocyte quality (ii) Improved embryos quality (iii) Improved pregnancies rate
Papaleo et al. 2009 [70]	Human	Orally administered MI + FA (2 g + 200 mg/twice daily) versus FA (400 mg/day)	60	30/30	IVF-ET ICSI	(i) Reduced number of immature oocytes (ii) Decreased FSH unit and day of stimulation (iii) Decreased E2 level before hCG stimulation

MI: myo-inositol; FA: folic acid; E2: estradiol; DCI: D-chiro-inositol; FF: follicular fluid; IVF: in vitro fertilization; ET: embryo transfer; ICSI: intracytoplasmic sperm injection; FSH: follicle stimulating hormone; hCG: human chorionic gonadotropin.

Chiu et al. [48] divided into two groups, according to oocytes quality, the oocytes retrieved from women treated with IVF: in group A they included good quality oocytes, whereas in group B they included bad quality oocytes. They showed that group A had significantly higher FF volume and higher MI level in FF without any difference in MI serum level between the two groups; MI was found to be higher in FF with oocytes that developed embryos with good morphology, with a positive correlation between MI level and fertilization rate. Also the estradiol level was higher in FF than in serum and was positively correlated with MI level in FF. Based on this evidence, a higher concentration of MI in FF could be due to the active transport of MI into FF as previously shown in an animal model [67]. These data suggest that MI is required to enhance the development of the maturing oocyte [48]. Other authors have shown that women under 40 years old with at least one of following features: a previous failed attempt with ICSI with low quality oocytes, or PCOS, or a diagnosis of "poor responders," treated with MI plus folic acid had higher number of good quality oocytes versus patients treated with only folic acid. The number of grade A embryos significantly increased the clinical pregnancy

Table 3: Effects of myo-inositol + melatonin supplementation during ART.

Authors	Species	Myo-inositol + melatonin in vitro/in vivo	Patients number (tot)	Intervention/control number	Technique	Significant outcomes ($p < 0.05$)
Pacchiarotti et al. 2016 [73]	Human	Orally administered MI (4 g/day) + FA (400 mcg/day) + M (3 g/day) versus MI (4 g/day) + FA (400 mcg/day) versus FA (400 mcg/day)	526	165/166/195	ICSI IVF-ET	(i) Increased mature oocytes (ii) Increased embryos grade 1 (iii) Reduced days of FSH stimulation (iv) Reduced E2 level before hCG
Unfer et al. 2011 [75]	Human	Orally administered MI (4 g/day) + FA (400 mcg/day) + M (3 g/day) versus previous failed IVF (themselves)	46	46/46	IVF-ET	(i) Increased mature oocytes (MII) (ii) Increased embryos grade 1 (iii) Reduced days and UI of FSH stimulation
Rizzo et al. 2010 [74]	Human	Orally administered MI (4 g/day) + FA (400 mcg/day) + M (3 g/day) versus MI (4 g/day) + FA (400 mcg/day)	65	32/33		(i) Increased mature oocytes (MII) (ii) Increased embryos grade 1-2

MI: myo-inositol; FA: folic acid; M: melatonin; IVF: in vitro fertilization; ET: embryo transfer; ICSI: intracytoplasmic sperm injection; FSH: follicle stimulating hormone; hCG: human chorionic gonadotropin; UI: uterine insemination.

rate without reaching a significant difference if clinical and biochemical pregnancy rates are considered [68]. Another study confirmed the positive role of MI plus folic acid supplementation versus only folic acid in patients with PCOS, increasing the number of oocytes recovered and increasing the numbers of ET and good quality embryos while at the same time a reduction of immature oocytes was observed [69, 70]. MI used in women with PCOS caused a reduction in the total number of units and number of days of FSH stimulation, reducing estradiol (E2) levels measured the day before human chorionic gonadotropin (hCG) administration, allowing a decreased risk of hyperstimulation syndrome [70]. Other authors compared MI with DCI in patients, finding that MI had the capacity to ameliorate the quality of oocytes, to decrease the number of immature oocytes, and to improve the number of good quality embryos and the total number of pregnancies compared to DCI treatment during an ovarian stimulation protocol [71]. Other authors [72] found that MI plus DCI improved the quality of oocytes and the quality of embryos, reducing immature oocytes compared to only DCI treatment both in women under and over 35 years old. In patients under 35 years old, MI plus DCI also improved the fertilization rate. These authors speculated that MI plus DCI together can more quickly improve "inositol imbalance" and that DCI alone should be avoided in the IVF technique. For a complete summary of the studies reported in this chapter, refer to Table 2.

6. Myo-Inositol and Melatonin in ART

Recently many authors have investigated whether supplementation with MI and melatonin (plus folic acid), administered together, improved the PCOS outcome in IVF. These data suggested a synergistic effect with both MI and melatonin, which were able to improve the number of MII and the number of grade I embryos (good quality) compared to MI alone [73, 74]. Furthermore, MI and melatonin reduced significantly the units of gonadotropin used for stimulation as well as the E2 level reached before hCG injection. There was no significant increase in pregnancy rates in the study group with respect to controls [73], although others [74] found that both the fertilization rate and the pregnancy rate tended to be higher in the group cotreated with melatonin. Based on these data, it is possible to speculate that the positive effect of melatonin as ROS scavengers and its effect on the increase in LH receptors and progesterone synthesis bound its receptor in granulosa-luteal cells resulting in luteinization and ovulation. MI also plays a crucial role in oocyte maturation and as an insulin-sensitizer molecule. These features of MI and melatonin lead to a positive effect on oocyte and embryo quality in IVF [73]. In addition, therapy with MI plus M in patients who failed to conceive in previous IVF cycles due to poor oocyte quality has been shown to improve oocytes and embryo quality during second IVF cycle, with a tendency toward augmented pregnancy rates, although it did not reach statistical significance in a large-cohort analysis [75]. For a complete summary of the studies reported in this chapter, refer to Table 3.

7. Conclusion

Melatonin is an oxidative scavenger and has an important role in the reduction of oxidative stress which physiologically increases during ovulation. This effect becomes more

important during IVF. The manipulation of oocytes, incubated with high oxygen levels, increases the oxidative stress. Studies in vitro and in vivo have shown that melatonin improved oocyte quality and embryo quality. Some studies have shown an increased fertilization rate, and in one study it increased the implantation rate. The pregnancy rate tended to increase, although it was not statistically significant. As expected, melatonin treatment increased the melatonin concentration in FF and reduced the oxidative stress in FF measured by the 8-OHdG level.

MI plays an important role in cytoskeleton and in chromatin stabilization, all necessary features for correct oocyte maturation. It plays an important role as a PtdIns precursor involved in the intracellular Ca2+ signaling pathway that has been shown to be fundamental to proper oocytes maturation. Furthermore inositols have an insulin-sensitizer effect. According to our overview, MI improves oocyte and embryo quality in patients undergoing IVF and is able to reduce units and days of FSH stimulation and E2 levels before hCG administration, thus reducing the risk of hyperstimulation syndrome especially in patients with PCOS and thus increasing pregnancy rates.

Combined therapy with MI and melatonin increased the positive effects described previously on outcome of ART. Cotreated patients showed an improved number of good quality oocytes and embryos, with reduced FSH units and days of treatment during cycles IVF.

Although these results are positive, more studies in large populations are necessary to confirm the data and to support the use of this supplementation therapy routinely in ART.

Competing Interests

The authors have no proprietary, financial, professional, or other personal interests of any nature in any product, service, or company. The authors alone are responsible for the content and writing of the paper.

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Clinical Study

A Potential Therapeutic Role of Myoinositol in the Metabolic and Cardiovascular Profile of PCOS Iranian Women Aged between 30 and 40 Years

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Introduction. Polycystic ovary syndrome (PCOS) is a common disorder in reproductive age. This pilot study investigated the effects of myoinositol (MI) treatment on metabolic and cardiovascular profile in PCOS women over 30 years of age. *Methods*. Between 2015 and 2016, 50 women with diagnosis of PCOS by the Rotterdam Criteria were included in the study. All women received MI 2 g plus 200 μg of folic acid (Inofolic, Health Parsian, Iran; twice daily) for 3 months. Baseline and 3-month serum samples were taken after an overnight fast to evaluate the insulin resistance index (HOMA-IR), fasting glucose, and the levels of triglyceride, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), homocysteine, systolic blood pressure, and diastolic blood pressure. Participants' weight was measured before and after treatment and body mass index (BMI) was calculated. *Results*. The data showed a significant improvement in the serum level of insulin sensitivity and a reduction of cholesterol, LDL, and homocysteine after three months of treatment. Furthermore, blood pressure was significantly reduced in the treated patients. Three participants became pregnant during treatment. *Conclusion*. Results showed that supplementation with MI and folic acid in PCOS patients over 30 years of age could decrease the risk of cardiovascular problems by normalizing the metabolic profile.

1. Introduction

Polycystic ovary syndrome (PCOS) is a common disorder in reproductive age which affects up to 20% of women. Polycystic ovaries on ultrasound, menstrual irregularity, and hyperandrogenism which can lead to acne, alopecia, hirsutism, insulin resistance, android obesity, dyslipidemias, infertility, and early pregnancy loss characterize it [1–3]. The main alteration induced by PCOS seems to be an increased androgen synthesis and secretion at ovarian level; insulin resistance and obesity may exacerbate this alteration, explaining the link between PCOS and obesity and insulin resistance [4]. Insulin resistance is a key factor not only in obese women but also in more than half of normal-weight women, indicating that, in the most severe cases, metabolic syndrome (MS) may be already established: this pathway can be responsible for the changing picture of PCOS throughout the life. These new findings imply that PCOS itself cannot be considered anymore as a typical disorder of young and fertile age, but it can be considered as an important condition in the different phases of life, from adolescence to postmenopause [5].

Indeed, more than half of women with PCOS suffer from insulin resistance, a condition that could cause the development of other related diseases such as MS, obesity, gestational diabetes, type 2 diabetes, and cardiovascular disease (CVD). Due to all the possible implications that PCOS can have in the medium and long term, great attention has been focused on the management of PCOS women as patients at risk of severe cardiovascular and metabolic diseases. The prevalence of at least one feature of MS has been found to be more than 50% in adult PCOS women, commonly constituted by

lipid alterations, such as HDL decrease and LDL and total cholesterol increase. Furthermore, typical signs of increased CVD risk are present (increase of systolic pressure, increase of diastolic pressure, etc.) [4, 5].

Hence, the clinical management of PCOS women and prevention of MS and CVD are important aspects in medicine [6–8], especially in those patients over 30 years of age.

Hormonal contraceptives, insulin-sensitizing drugs such as metformin, thiazolidinediones, statins, orlistat, and N-acetylcysteine treatments were performed for women with PCOS but their usage is limited due to some contraindications and side effects [9–13].

Recent studies have proven that alteration in the insulin pathway could be due to defected inositolphosphoglycans (IPGs) second messengers. IPGs are directly involved in activating the glucose metabolism; therefore, PCOS women seem to show a defect in tissue availability or altered metabolism of inositol that could bring about insulin resistance [14]. MI is the most abundant isoform of inositol in nature and in the human body and it belongs to the vitamin B complex group with insulin-like action. It is hypothesized that there is a correlation between reduction of MI and insulin resistance. Several studies suggested the efficacy of MI in reducing the insulin resistance and improving the ovarian function in PCOS women, but its efficacy to ameliorate the cardiovascular and metabolic profile of adult PCOS women has not been fully elucidated [14–16].

The aim of this pilot study was to investigate the effects of MI treatment based on the cardiovascular and metabolic profile of PCOS Iranian women.

2. Methods

Fifty women in reproductive age, between 30 and 40 years, with diagnosis of PCOS by Rotterdam's criteria (European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine; ESHRE/ASRM 2003) were enrolled into the study in the gynecologic clinic of Taleghani Hospital between 2015 and 2016. All participants signed a written informed consent. Ethical committee of Shahid Beheshti University of Medical Sciences (SBMU) approved the study.

Exclusion criteria were absence of enzymatic adrenal deficiency and/or other endocrine diseases and no hormonal treatments in the previous 6 months.

All the women received MI 2 g plus 200 μg of folic acid twice daily (Inofolic, Health Parsian, Iran) for 3 months. Patients were not instructed to follow any diet or lifestyle modification. Baseline and 3-month serum samples were taken after an overnight fast to determine level of fasting glucose, insulin resistance (HOMA), homocysteine, triglyceride, total cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL). Systolic pressure and diastolic pressure were also checked at baseline and after treatment in all the patients. Participants' weight was measured before and after treatment and BMI was calculated.

Insulin sensitivity was calculated as glucose-to-insulin ratio. HOMA index was calculated as [basal glucose] × [basal insulin]/22.5.

The homocysteine levels were detected using a fluorometric HPLC protocol.

Sample size was determined after consideration of type 1 statistical error <5% and type 2 statistical error <20%.

Results were shown as mean ± SD. Statistical analysis was performed using the SPSS 21.0 statistical software package (SPSS Inc., Chicago, IL, USA). Plasma triglycerides were analyzed with Mann–Whitney U test and other data were analyzed with t-test for quantitative variables and Chi square test for qualitative variables. A p value of 0.05 was considered significant.

3. Results

Fifty participants were enrolled in this pilot study. Forty-six participants were able to complete the study and their data were included in the final analysis. Four subjects refused the study as follows: one subject discontinued intervention because of dry mouth and three subjects became pregnant during the study.

Data are shown as mean ± SD. Table 1 provides a summary of the baseline characteristics and outcomes data after a treatment of 3 months.

Significant results were observed in all the patients for what concerned the hormonal and metabolic parameters under evaluation.

As shown in Table 1, there was a significant decrease in the fasting glucose, HOMA index, cholesterol, and LDL. For what concerned cardiovascular parameters, a significant decrease was observed in the homocysteine levels and in the levels of systolic pressure and diastolic pressure (Table 1).

Furthermore, no significant difference was found in the HDL and triglycerides levels. Even the BMI was not influenced by the treatment.

Among 38 participants with irregular menstrual cycles including oligomenorrhea, 60.5% had regular menstrual cycle after treatment. Three participants were pregnant spontaneously during the study just with MI intake and without any other intervention.

No relevant side effects were reported during and after the treatment among the patients.

4. Discussion

The present study shows that supplementation with MI and folic acid positively affects metabolic parameters (i.e., insulin sensitivity) and the cardiovascular profile of women over 30 years of age affected by PCOS.

PCOS is a common endocrine disorder in women in reproductive age. Ovarian dysfunction, androgen excess, insulin resistance, and obesity may increase the risk of infertility, type 2 diabetes, CVD, psychological disorders, and cancer. Most of the PCOS women present the typical signs of increased cardiovascular risk and metabolic disorders. In particular, obesity itself has a significant impact on the severity

Table 1: Patients' characteristics and outcomes data.

	Before treatment	After treatment	p value
Fasting glucose (mg/dL ± SD)	82.06 ± 4.7	79.46 ± 4.2	0.011*
HOMA IR	3.4 ± 2.5	2.6 ± 2.5	0.001*
Homocysteine (μmol/L)	9.10 ± 2.6	8.54 ± 2.39	0.008*
Cholesterol (mg ± SD)	174.7 ± 41.0	160.3 ± 29.5	0.005*
LDL (mg ± SD)	94 ± 26.5	86.3 ± 18.3	0.017*
HDL (mg ± SD)	47.7 ± 6.5	48.6 ± 6.9	NS
Triglyceride (mg ± SD)	108.2 ± 55.3	110.7 ± 55.3	NS
Systolic blood pressure (mmHg)	130 ± 1.9	126 ± 1.9	0.002*
Diastolic blood pressure (mmHg)	89 ± 1.1	83 ± 3.1	0.001*
BMI (kg/m^2 ± SD)	26.9 ± 3.4	26.8 ± 3.4	NS

*Statistically significant differences was observed.
HOMA IR: insulin resistance; LDL: low-density lipoprotein; HDL: high-density lipoprotein; BMI: body mass index; SD: standard deviation.

of these manifestations. Therefore, adequate management in PCOS is necessary to decrease the morbidities [17–19].

Not only is PCOS relevant during the young and fertile age, but also elder women can present some related signs and symptoms, which could predict an increased risk of MS, with sequent manifestations during the menopausal period [5].

MI, previously classified as belonging to the vitamin B class, is commonly used in PCOS treatment without any reported and relevant side effects [20]. The efficacy of MI in reduction of insulin resistance, hirsutism, and hyperandrogenism and improvement of ovarian function was reported in several studies but only a few of them have directly checked the outcome in nonyoung PCOS women, in particular for what concerned the efficacy on the lipid profile and cardiovascular profile [14, 15, 21, 22].

Papaleo and coworkers designed a trial to determine the effects of MI on oocyte quality in PCOS women undergoing intracytoplasmic sperm injection (ICSI) cycles. The data showed the reduction of degenerated oocyte and germinal vesicles at ovum pick-up after MI treatment [23].

Gerli and coworkers reported a significant increase in HDL level after MI treatment in PCOS women but the reduction of LDL level was not significant [24].

Furthermore, very recent evidence has been published regarding the positive role of inositol in the metabolic profile of PCOS. An international consensus conference confirmed that MI is effective in the restoration of insulin signaling and some other important parameters negatively influenced by this syndrome [25].

Moreover, besides MI, a great interest in some natural agents has been increased in the recent years. In particular, cocoa polyphenols have shown a strong beneficial action on the cardiovascular profile of different models [26, 27]. Interestingly, there is some evidence showing that the combination of MI and cocoa polyphenols in postmenopausal women with MS was able to restore their metabolic and cardiovascular profile [28, 29]. Therefore, it could be speculated that PCOS women could take advantage of MI supplementation from the adolescence up to the menopausal age. This is the first trial of MI in PCOS women in our country. The prevalence of PCOS in Iranian population is around 15% [30]. We designed the trial to evaluate the efficacy of MI on the metabolic and cardiovascular profile of Iranian PCOS women over 30 years of age. Our data suggested that MI is effective in improving the insulin sensitivity, the lipid parameters, and the blood pressure after a short treatment of three months. However, MI treatment did not influence the BMI of the patients; therefore, we could speculate that restoration of the metabolic profile is mainly due to the insulin-sensitizing action of MI and not due to a different diet or lifestyle during the treatment period. Hence, long-term administration of MI may be helpful to decrease the risk of serious CVD with a possible improvement of fertility without parallel administration of hormonal treatments.

5. Conclusion

It seems that use of MI plus folic acid in PCOS patients aged between 30 and 40 years could decrease the risk of cardiovascular and metabolic problems by normalizing the insulin, the lipid profile, and the blood pressure profile. Anyway, further studies with longer treatments and bigger population should be conducted to prove the use of this new agent in these target patients. If further evidence will be assessed, myoinositol combined with folic acid might be considered to be one of the choices for the treatment of PCOS women at risk of metabolic syndrome and consequently for the reduction of their cardiovascular risk.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Review Article

Inositols in the Treatment of Insulin-Mediated Diseases

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A growing body of research is currently focused on the role of inositol isomers and in particular myo-inositol (MYO-INS) and D-chiroinositol (DCI) in the treatment of insulin resistance states. Both isomers have been shown to exert insulin-mimetic action and to lower postprandial glucose. Further, insulin resistance-related diseases were associated to derangements in inositol metabolism. Thus, the aim of this review is to provide current evidence on the potential benefits of inositol isomers (MYO-INS and DCI) in the treatment of disease associated to insulin resistance such as polycystic ovary syndrome (PCOS), gestational diabetes, and metabolic syndrome. Finally, molecular insights into inositol insulin-sensitizing effects will be covered focusing on the possible role of inositol glycans as insulin second messengers.

1. Introduction

Inositol (Ins), also known as cyclohexane-1,2,3,4,5,6-hexol, belongs to vitamin B complex. Inositol is a polyol that thanks to epimerization exists under nine stereoisomeric forms depending on the spatial orientation of its six hydroxyl groups. Myo-inositol (MYO-INS) and D-chiroinositol (DCI) are stereoisomers of inositol that are currently used for the treatment of polycystic ovary syndrome (PCOS) [1, 2]. 99% of Ins is present in both nature and mammalian cells under MYO-INS form while the remaining 1% is present under DCI form. MYO-INS regulates glucose metabolism and transport [3]. MYO-INS could be found both in free form and as component of the membrane phosphoinositides into the cells [4]. Phosphatidyl-myo-inositol is the precursor of phosphatidyl-Ins phosphate and phosphatidyl-Ins bisphosphate (PIP2) whose hydrolysis results in Ins trisphosphate (Ins-1,4,5P3, InsP3) that plays the role of second messenger taking part in the activities of several hormones such as follicle-stimulating hormone (FSH) and insulin [4, 5]. Moreover, InsP3 binds the membrane receptors of mitochondria and the endoplasmic reticulum, leading to an increase in calcium influx into the cytosol, which activates protein kinase C and mediates cellular responses [4].

MYO-INS plays the role of precursor of phosphoinositides, membrane components, second messenger signalling, and hyperosmotic stress protectant in both germ and somatic cells [6, 7]. The conversion of MYO-INS into DCI is made by NAD/NADH epimerase that is an insulin-dependent enzyme whose activity is the major determinant of the concentrations of these two molecule in hepatic, adipose cells and muscle fibers [8]. The epimerization of MYO-INS is particularly relevant to hepatic and muscle cells, taking part in glycogen synthesis [3].

The role of DCI in insulin signalling pathway is expressed in terms of stimulation of pyruvate dehydrogenase phosphatase, protein phosphatase 2C, and Ins-phosphate glycan [9].

The blunted ratios of increased MYO-INS to decreased DCI in urine have been indicated as marker of insulin resistance in human subjects [8]. In fact, muscle biopsies of diabetic subjects revealed the absence of DCI compared

to controls, even after insulin administration and increased levels of MYO-INS, which was further induced by insulin administration [10]. A decrease of 50% of DCI has been found in both urine and hemodialysate of diabetic subjects [11]. It has been hypothesized that this imbalance between MYO-INS and DCI could be due to a defect in MYO-INS to DCI epimerization. In agreement with this hypothesis, a reduction of [^{3}H]-MYO-INS to [^{3}H]-DCI has been demonstrated in muscle, liver, and fat cytosolic extracts of Goto-Kakizaki diabetic rats compared to Wistar control rats [12]. In the same tissues a 2-fold decrease of epimerase activity has been observed and this was consistent with human evidence [10]. The reduction of DCI results in the decreased availability of inositol phosphoglycan that is a second messenger of insulin and thus contributing to the onset of insulin resistance.

The aim of this paper is to review the current evidence regarding the role of MYO-INS and DCI in the pathogenesis and treatment of diseases characterized by insulin resistance.

2. PCOS

PCOS is characterized by reproductive and metabolic implications and it is currently considered as the most common cause of female infertility [13, 14]. In particular PCOS has been associated with several risk factors for cardiovascular disease (CVD) such as obesity, diabetes, hypertension, and dyslipidemia which share the same pathogenetic mechanism, that is, insulin resistance [14–16].

Insulin resistance and compensatory hyperinsulinemia result in an increased ovarian androgen production along with the reduction of hepatic sex hormone binding globulin (SHBG) production. This derangement is responsible for androgen excess [27]. Obesity may contribute to worsening insulin resistance and as a consequence the degree of androgen excess, the reproductive features of PCOS, and the clinical PCOS phenotypes. Insulin resistance has been demonstrated to be an intrinsic feature of PCOS. A clamp study performed in both lean and overweight PCOS women and controls demonstrated that insulin resistance was independent of BMI in PCOS and was present in 85% of PCOS women and, in particular, in 75% of lean PCOS women and 95% of overweight PCOS women [28, 29]. Further, the adverse impact of obesity on insulin resistance was greater in PCOS women than controls. As a matter of fact overweight control women had comparable degree of insulin resistance to lean PCOS women, suggesting that PCOS women are metabolically similar to overweight/obese non-PCOS women [28].

The major defect of insulin action in PCOS patients is probably due to a postbinding defect in insulin receptor-mediated signal transduction that results in a significant derangement in receptor binding [30]. This seems to be an intrinsic genetic defect in PCOS women and consists of increased insulin-independent serine phosphorylation and decreased insulin-dependent tyrosine phosphorylation [30]. Insulin resistance and compensatory hyperinsulinemia might contribute to the spectrum of the disorders related to PCOS by different mechanisms. Insulin may exert its action on pituitary regulating LH secretion, although studies performed on this topic provided conflicting results [30]. Rat pituitary cells preincubated with insulin showed an increased response of LH after GnRH administration while insulin infusion in PCOS women did not change LH secretion or release after GnRH stimulation [30]. Further, the experimental reduction of hyperinsulinemia has been reported to decrease serum LH levels [30], although it is unknown whether decreased serum LH is an effect of decreased insulin levels or of increased ovarian estrogen production due to resumed folliculogenesis. At the peripheral level, insulin resistance acts on hepatic, muscle, and ovarian function. Hyperinsulinemia decreases the hepatic synthesis of SHBG and this mechanism contributes to the increase of the free androgens and consequently the peripheral androgen action; moreover, hyperinsulinemia also blocks the hepatic secretion of the IGF binding protein-(IGFBP-) 1, resulting in an increased bioactivity of IGF-I and IGF-II which are two important regulators of ovarian follicular maturation and steroidogenesis [30]. The ovarian androgen production from theca cells is increased by IGF-I and IGF-II systemic increase that binds IGF-I receptors [30]. The decreased expression of genes involved in mitochondrial oxidative metabolism has been reported at muscular level [31]. Alterations in insulin signaling pathways along with free fatty acid (FFA) metabolism also have been detected at muscular level in insulin resistance state [31]. Hyperinsulinemia prevents follicular development and causes anovulation through two mechanisms: (1) directly acting at ovarian level causing premature follicular atresia and antral follicular arrest [30] and (2) indirectly causing dysfunction of ovarian response to endogenous gonadotropins [30]. In fact women at the time they present complaining of infertility and/or irregular menses already showed adverse metabolic effects of PCO. Obesity has been reported to worsen menstrual irregularity and increase the follicle number and serum total testosterone level [32, 33].

Oral contraceptives (OCs) are considered the first-line treatment for menstrual disturbances and hirsutism/acne in patients with PCOS [34, 35], while the use of oral insulin-sensitizing compounds such as pioglitazone and metformin is suggested for the treatment of hyperinsulinemic condition of PCOS patients [36]. In particular metformin is the most used insulin-sensitizing drug in the treatment of PCOS. Metformin enhances insulin sensitivity inhibiting hepatic glucose production in liver and increasing glucose uptake and utilization in muscle tissue and decreases total and free-testosterone concentrations. However, metformin can induce gastrointestinal side effects, thus decreasing patients' compliance [37]. Further, metformin has been reported to have a beneficial effect in cancer treatment. Patients with early stage breast cancer who were receiving metformin along with neoadjuvant therapy experienced a higher pathologic complete response [38]. Metformin usage was also associated with improved survival of diabetic prostate cancer patients [39]. The possible molecular mechanism for the anticancer effect of metformin depends on the stimulation of AMP-activated protein kinase (AMPK) and its upstream regulator liver kinase B1 (LKB1) that is a well-known tumor suppressor protein [40]. Inositol has also been reported to have an anticancer effect both on prostate and on human colon

Table 1: Clinical studies in which MYO or DCI supplementation has been evaluated for the treatment of disease associated to insulin resistance.

Authors, year	Inositol isomers	Duration of treatment	Experimental models
Genazzani et al., 2008 [17]	MYO 2 gr	12 weeks	PCOS
Genazzani et al., 2012 [18]	MYO 2 gr	8 weeks	PCOS
Gerli et al., 2007 [19]	MYO 4 gr	14 weeks	PCOS
Artini et al., 2013 [20]	MYO 2 gr	12 weeks	PCOS
Iuorno et al., 2002 [21]	DCI 600 mg	6 to 8 weeks	PCOS
Laganà et al., 2015 [22]	DCI 1 gr	6 months	PCOS
Giordano et al., 2011 [23]	MYO 2 gr BID	6 months	Postmenopausal women with metabolic syndrome
Santamaria et al., 2012 [24]	MYO 2 gr BID	12 months	Postmenopausal women with metabolic syndrome
Corrado et al., 2011 [25]	MYO 4 gr	8 weeks	Pregnant women with gestational diabetes
D'Anna et al., 2013 [26]	MYO 2 gr	6 months	Pregnant women with a family history of type 2 diabetes

Note. MYO: myo-inositol; DCI: D-chiroinositol.

cancer [41, 42]. The mechanism by which Ins exerts an anticancer effect seems to be related to the ability of inositol to decrease the mRNA and protein expression of PI3K and Akt. Moreover, Ins inhibited the phosphorylation of Akt (pAkt), whereas it increased the expression of its downstream effector, caspase-9, thus suggesting that inositol suppresses cell survival and proliferation by targeting PI3K/Akt pathway [42].

Recently MYO-INS and DCI have been used for the treatment of PCOS. MYO-INS has also been reported to significantly decrease hyperandrogenism ($p < 0.001$) and insulin resistance ($p < 0.001$) in women with PCOS [43, 44]. Further, MYO-INS has been demonstrated to restore spontaneous ovarian activity and thus fertility in most patients with PCOS [45]. A randomized controlled trial was performed in 20 overweight PCOS women that were randomized to receive MYO-INS 2 gr plus folic acid 200 mug or only folic acid 200 mug. Patients taking MYO-INS experienced a significant improvement of reproductive axis ($p < 0.05$) and insulin resistance ($p < 0.05$) state after 12 weeks of supplementation [17]. On such basis, the efficacy of MYO-INS 2 gr for 8 weeks of treatment has been investigated in 42 PCOS obese women. Although all the enrolled subjects improved both hormonal and insulin resistance parameter, PCOS women with fasting insulin levels above 12 μU/mL experienced a greater reduction of both fasting insulin plasma levels and area under the curve of insulin under oral glucose tolerance test compared to patients with fasting insulin levels below 12 μU/mL [18]. A significant weight reduction ($p < 0.01$) along with decrease in leptin levels ($p < 0.01$) has been reported in a double-blinded, placebo-controlled study in which 92 women were randomized to receive 400 mcg folic acid as placebo or MYO-INS plus folic acid (4 g MYO-INS plus 400 mcg folic acid) for 14 weeks of treatment [19]. Artini et al. provided evidence that 12 weeks of treatment with MYO-INS were effective in reducing plasma LH ($p < 0.005$), prolactin ($p < 0.05$), LH/FSH ($p < 0.01$), and insulin resistance measured by HOMA-IR index ($p < 0.01$) [20]. Unfer et al. [2] reviewed and analyzed the six Randomized Controlled Trials (RCTs) that assessed the effectiveness of MYO-INS supplementation in improving PCOS hormonal and metabolic disturbances. A dosage of 2 and 4 g/day was tested for 12 and 16 weeks in those studies and no side effects were reported. They provided level Ia evidence of MYO-INS effectiveness that was mainly based on improving insulin sensitivity of target tissues. This mechanism has a beneficial effect on the reproductive axis, restoring ovulation and improving oocyte quality, and on hyperandrogenism.

DCI has been reported to reduce insulin resistance both in lean and in obese patients with PCOS concurring to improve ovarian function and hyperandrogenism [1, 21]. A retrospective study has been performed in patients with irregular cycles showing that treatment with DCI improves indexes of insulin resistance along with an increase of the percentage of women reporting regular menstrual cycles which was directly proportional to the increased duration of DCI treatment (24% and 51.6% at a mean of 6 and 15 months of treatment) [46]. One gr of DCI/die plus 400 mcg of folic acid daily *per os* for 6 months significantly improved insulin resistance as measured by HOMA Index ($p = 0.001$) and glycemia/insulin resistance index (IRI) ratio ($p = 0.001$). In the same study an improvement of systolic blood pressure ($p = 0.001$), Ferriman-Gallwey score ($p = 0.001$), LH ($p = 0.001$), LH/FSH ratio ($p = 0.001$), total testosterone ($p = 0.001$), free testosterone ($p = 0.001$), Δ-4-Androstenedione ($p = 0.026$), Prolactin ($p = 0.010$), and sex hormone binding globulin ($p = 0.001$) has been reported [22] (Table 1).

Based on the current evidence, it is widely accepted that both MYO-INS alone and DCI alone and their combination may have a beneficial effect on metabolic derangements associated to PCOS. Although there is no robust consensus regarding the ideal dosage of MYO-INS and/or DCI for PCOS treatment, a combination of both MYO-INS and DCI has been suggested by the promising results from studies with their combination [47]. The "International Consensus Conference on myo-inositol and D-chiro-inositol in Obstetrics and Gynecology" [48] suggests administering MYO-INS and DCI in a proposed "physiological ratio" of 40 : 1 based on the assumption that plasma ratio of MYO-INS/DCI in normal subjects is 40 : 1. However, the "International Consensus Conference on myo-inositol and D-chiro-inositol in Obstetrics and Gynecology" also suggests not giving high dose of DCI

for the treatment of PCOS in order to avoid the negative effect of high dose of DCI on the ovary (the so-called DCI paradox).

3. Inositol and Other Insulin Resistance States

MYO-INS has been administered to postmenopausal women in order to investigate the effect on metabolic parameters. A prospective study assessed the effect of MYO-INS 2 g BID plus diet in postmenopausal women reporting a significant decrease of 75% of HOMA-IR index along with other metabolic markers (triglycerides, HDL cholesterol, and diastolic blood pressure) [23]. Patients were randomized to receive MYO-INS 2 g BID or placebo for 12 months. At the end of the study there was an improvement of all the metabolic parameters such as glucose, insulin, HOMA-IR (Homeostasis Model Assessment-Insulin Resistance), triglycerides, total and high density lipoprotein cholesterol, body mass index (BMI), waist circumference, and blood pressure [24]. Pregnant women with gestational diabetes were randomized to receive MYO-INS supplementation (4 g daily) plus folic acid (400 μg daily) or folic acid only (400 μg daily) as supplement to controlled diet for 8 weeks. Fasting glucose ($p < 0.05$), insulin ($p < 0.05$), and consequently HOMA-IR ($p < 0.05$) index significantly improved in both groups, although the improvement was greater in women treated with MYO-INS. Further, the treatment with MYO-INS was accompanied by an increase in adiponectin levels, while there was a decrease of adiponectin levels in the control group [25]. A two-year, prospective, randomized, open-label, placebo-controlled study was carried out on pregnant outpatients with a family history of type 2 diabetes who were randomized to receive 2 g MYO-INS plus 200 μg folic acid or only 200 μg folic acid twice a day from the end of the first trimester. MYO-INS supplementation significantly reduced the incidence of gestational diabetes ($p = 0.04$) and the delivery of macrosomia fetuses ($p = 0.007$) in pregnant women with a family history of type 2 diabetes [26] (Table 1).

Human evidence also has been confirmed by basic studies. The administration of sequoyitol, that is, the 5-O-methyl form of MYO-INS, (80 mg/kg per day) for 8 and 10 weeks, had antidiabetic effects in mice when administered chronically. In fact, both subcutaneous and oral administrations of sequoyitol improved glucose derangements and enhanced insulin signaling in liver of ob/ob insulin resistant mice [49]. The reduction of glucose levels after glucose load has been reported in healthy mice after both acute [50] and chronic administration of MYO-INS [51].

This effect was due to an improvement in peripheral insulin sensitivity that has been observed *in vivo* during an insulin tolerance test along with an enhanced GLUT-4 translocation to the plasma membrane in response to hyperglycemia at the skeletal muscle level [50].

4. Conclusion

The insulin-mimetic properties of dietary inositol are due to the production of inositol glycan secondary messengers containing either MYO-INS or DCI. Although randomized control trials using MYO-INS and/or DCI as supplement gave positive results in improving insulin resistance and reducing cardiovascular risk factors in women with PCOS and gestational diabetes mellitus or metabolic syndrome postmenopause, larger studies including both genders are needed in order to extend a possible application for a more generalized population at risk of developing or already presenting insulin resistance.

Competing Interests

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

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Research Article

Myoinositol as a Safe and Alternative Approach in the Treatment of Infertile PCOS Women: A German Observational Study

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The use of 2 × 2000 mg myoinositol + 2 × 200 μg folic acid per day is a safe and promising tool in the effective improvement of symptoms and infertility for patients with a polycystic ovary syndrome (PCOS). Using a questionnaire an observational study was performed under German gynecologists to collect data on ovulation and pregnancy rates in PCOS patients with infertility. In this observational study, 3602 infertile women used myoinositol and folic acid between 2 and 3 months in a dosage of 2 × 2000 mg myoinositol + 2×200 μg folic acid per day. In a subgroup of 32 patients, hormonal values for testosterone, free testosterone, and progesterone were analyzed before and after 12 weeks of treatment. The mean time of use was 10.2 weeks. During this time 70% of these women had a restored ovulation, and 545 pregnancies were obtained. This means a pregnancy rate of 15.1% of all the myoinositol and folic acid users. In 19 cases a concomitant medication with clomiphene or dexamethasone was used. One twin pregnancy was documented. Testosterone levels changed from 96.6 ng/ml to 43.3 ng/ml and progesterone from 2.1 ng/ml to 12.3 ng/ml ($p < 0.05$) after 12 weeks of treatment. No relevant side effects were present among the patients. This study could show that a new treatment option for patients with a PCOS and infertility is available. The achieved pregnancy rates are at least in an equivalent or even superior range than those reported by the use of metformin.

1. Introduction

The PCOS is the most common cause of menstrual disorders, ovarian dysfunction, and infertility of women. Observational studies postulate that up to 15% of women suffer under this condition during their reproductive life. PCOS etiopathology is not clear, but most probably a strong genetic cause that is influenced by gestational environment and lifestyle seems to be the key factor. The most common features of PCOS are hyperandrogenism, chronic anovulation, typical PCOS ultrasound images, and skin issues such as acne, hirsutism, and seborrhea. Furthermore, recently it has been found that insulin resistance plays a key role in the clinical development of PCOS in almost all the women. Severe disorders of the insulin sensitivity with a compensatory hyperinsulinemic state not only in obese PCOS patients but also in lean women have been described, so that the hypothesis is strongly supported that the insulin resistance is independent of the weight [1]. In particular, the related hyperinsulinemia could induce an excess of androgens production in PCOS women through two different ways: first one is direct stimulation of ovaries to produce androgens, and the other one is the reduction of sex hormone binding globulin (SHBG) serum levels [2].

Due to the key role of insulin in the syndrome etiopathology, for many years, insulin sensitizers such as metformin, pioglitazone, or troglitazone have been considered as possible therapeutic options in the management of these problems. Metformin has been used in the last time on patients with a hyperinsulinemic status for the improvement of ovarian dysfunction with consecutive anovulation, irregular menstrual cycles, and infertility problems [3, 4]. Nevertheless

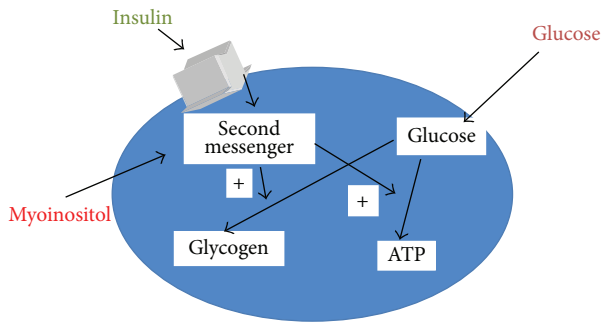

Figure 1: Mechanism of action of myoinositol in the cell.

metformin, when used in the therapeutic dose range, was shown to have several side effects such as flatulence, diarrhea, and nausea, so that many patients are unable to use this treatment option in gynecology for a longer period of time [5, 6].

Therefore, in parallel to the common use of metformin and other insulin sensitizer agents for the treatment of PCOS, in the recent years, other therapeutic alternatives have been investigated.

Myoinositol is one of the most interesting molecules that have been studied for the treatment of PCOS.

The substance inositol is a *chemical compound* with the formula $C_6H_{12}O_6$ or $(–CHOH–)_6$, a sixfold *alcohol* (*polyol*) of *cyclohexane*, with five equatorial and one axial hydroxyl group. It is widely found in nature. There exist nine different stereoisomer forms, but myoinositol is the most common one found in nature. In fact, myoinositol is very often found in many plants and in tissues of animals. The second most common form is D-chiro-inositol. It is important to distinguish between the lecithin formulation that is bioavailable for the human and the phytate formulation of corns that are not bioavailable. Foods with the highest concentration of myoinositol are fruits, beans, corns, and nuts [7].

Inositol was defined in the past as "myometrial sugar," but it is indeed not a substance belonging to the carbohydrate group if we use modern definitions. Defining inositol as a vitamin B is also being discussed with controversy as inositol is not an essential substance and it can be produced in human cells from glucose [8]. In fact, several studies have proved that the inositol molecule is directly involved in the insulin cellular signaling.

Regarding PCOS, several studies have shown that one of the mechanisms of insulin deficiency has its rise from the inositolphosphoglycan (IPG) mediator and that a deficiency of inositol in the inositolphosphoglycans is responsible for insulin resistance. It has demonstrated that the administration of D-chiro-inositol (intracellularly converted from myoinositol) could reduce the insulin resistance [9] (see Figure 1).

Indeed, myoinositol, as a second messenger, plays an essential role for the signal pathways of cells. In particular, the action of myoinositol in a PCOS pathway would be related to an improved insulin sensitivity and a sequent increased intracellular glucose uptake [2, 10].

All these pieces of evidence have opened a new clinical interest on myoinositol, as a potential insulin sensitizer agent to be used as safe and effective option in PCOS patients, through the restoration of their metabolic profile and a consequent ovulation induction in infertile PCOS patients. Studies report also a very good safety profile of the molecule, even when administered up to 12 grams/day, where only mild gastrointestinal side effects have been reported [11].

The aim of this study was to determine the pregnancy rates under the use of a combination of myoinositol and folic acid in patients with a PCOS in Germany, to establish if this molecule can be used as a safer treatment option for the fertility improvement of this disease.

2. Patients and Methods

A standardized questionnaire was created and a questionnaire (see Appendix) was presented to 245 gynecologists present in Germany, between June 2014 and March 2015. During this time reports were generated of 3602 women with a PCOS and infertility according to the Rotterdam classification. The women started with the intake of myoinositol and folic acid at a dosage of 2×2000 mg myoinositol and $2 \times 200\,\mu$g folic acid per day and used it for at least 2-3 months. The primary outcome of the study was to determine the ovulatory function restoration and the pregnancy rate after treatment. The pregnancies were documented by the gynecologists and registered in a database, and these women were followed up during the whole pregnancy. Secondary outcome was the evaluation of side effects reported in those patients undergoing treatment. In a subgroup of patients, hormonal values were also evaluated. The values investigated were testosterone, free testosterone, and progesterone. In this group of patients the pregnancies outcome has also been checked.

3. Results

The data of 3602 patients with a PCO syndrome were evaluated. According to the obtained records 2520 women experienced an improvement of their menstrual cyclicity towards ovulatory cycles. Among them, a total number of 545 women became pregnant. The pregnancies occurred after the intake of two to three months of mayoinositol and folic acid. This means a ratio of 15.1% of the investigated women becomes pregnant during this observational study. No twin pregnancies were documented.

No relevant side effects have been reported in the patients taking myoinositol and folic acid product.

Figure 2 depicts the data. In the subgroup of 32 patients where hormonal values were evaluated a significant improvement of androgen levels and a rise in the progesterone values were observed.

This is shown in Table 1. The Appendix depicts the used German questionnaire. Furthermore, out of these 32 women who became pregnant, 5 of them experienced an abortion, whereas the remaining 27 delivered healthy newborns.

Table 1: Hormonal data before and after treatment with myoinositol.

	Total testosterone (ng/dL)	Free testosterone (ng/dL)	Progesterone (ng/mL)
Before treatment	96.6 ± 7.5	1.2 ± 0.7	2.1 ± 0.6
After treatment	43.3 ± 5.3	0.35 ± 0.1	12.3 ± 1.3

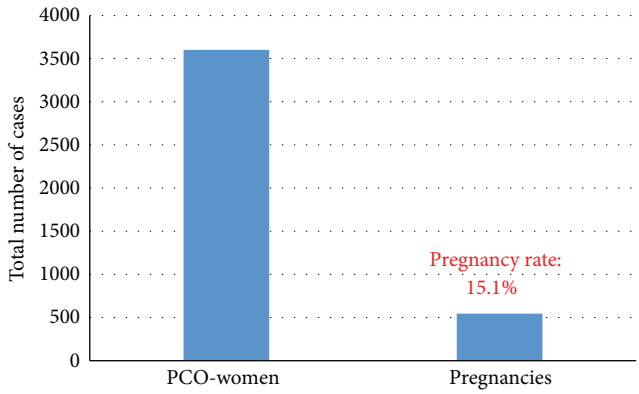

Figure 2: Number of patients and pregnancy rates.

4. Discussion

Despite the clear limitations of the observational study, there are reliable available data, since a wide range of patients can be analyzed. This study could show that a new treatment option for patients with a PCOS and infertility is available. Seventy % of the patients restored ovulation after the treatment. Furthermore, the achieved pregnancy rates are at least in a range equivalent to or even superior to those reported by the use of the insulin sensitizer metformin. Karimzadeh and Javedani [12] described a pregnancy rate of 14.4% in a cohort of 90 women and Legro et al. [13] of 12.3% in a cohort of 75 women with PCOS.

The interesting results that the study has shown seem to be related to the mechanism of action of myoinositol. The administration of this molecule, acting as a direct messenger of insulin signaling and improving the glucose tissues uptake, could improve the insulin resistance status of PCOS women, restoring indeed their hormonal status and restoring the ovulation process.

Another important evidence is also related to the difference of myoinositol and metformin in terms of safety profile and compliance for patients. In patients under metformin, side effects have been commonly reported, in particular from mild up to severe gastrointestinal side effects, such as abdominal pain, nausea, and diarrhea. Only in rare cases, very severe side effects as lactic acidosis have been found. On the other side, myoinositol seems to be a safe and well-tolerated approach, anyhow able to give similar results of metformin in terms of clinical efficacy.

In fact, many studies have demonstrated in the last months that an improvement in the rates of ovulation and regularization of menstrual cycles was obtained by the combined use of 4 g myoinositol with 400 μg folic acid per day. Gerli et al. [14] could show in a prospective study that the group of patients receiving myoinositol + folic acid experienced in 82% of the cases an ovulation, whereas this was only observed in 63% of the cases in the group of patients which received a placebo. By the same way 70% of the patients of the myoinositol group developed regular menstrual cycles after 16 weeks of treatment, whereas only 13% of the women did it in the placebo group.

In a study of Raffone et al. [15], where a comparison between the administration of myoinositol (2 × 2000 g + 200 μg per day) and the administration of metformin (1500 mg per day) in women with a PCO syndrome was performed, it could be shown that the number of pregnancies was clearly higher in the myoinositol group than in the metformin group of patients.

Some other studies upon others have shown the efficacy of myoinositol in the improvement of the fertility of PCOS patients due to its improvement of the insulin resistance of these women [16–18].

Many studies have been performed that show that the treatment with myoinositol + folic acid in the classical dosage (2 × 2000 g myoinositol + 200 μg folic acid per day) leads to significant positive changes of metabolic and hormonal parameters. Costantino et al. [19] could show in a double-blinded, placebo controlled study that myoinositol led to a statistically significant improvement of the blood pressure, triglycerides, cholesterol, glucose, and insulin values after a 75 mg oral glucose tolerance test. These improvements were achieved after a treatment period of 16 weeks. The evaluated hormonal values showed a significant decrease of the total and free testosterone serum levels and at the same time the progesterone levels, as a marker of ovulation, experienced a significant rise in the group that received myoinositol (see Table 1). This could show that myoinositol did lead not only to positive changes in metabolic parameters but also to a reduction of elevated androgenic values and subsequently to an improvement of skin problems such as acne or hirsutism.

These data can be supported by our own data as a rise of progesterone from a value of 2.1 ng/mL to a value of 12.3 ng/mL could be observed. By the same time a reduction in the levels of testosterone (from 96.6 ng/mL to 43.3 ng/dL) and free testosterone (from 1.2 ng/mL to 0.35 ng/mL) could also be observed.

A meta-analysis of Unfer et al. [20] could validate these data. This study could also show that, under the investigated studies, where the dosage of 4000 g myoinositol + 400 mg folic acid was used, no side effects were observed, especially those which are seen when other insulin sensitizers like metformin are used in high levels of 1500 mg per day.

Improvement in ovulation induction with myoinositol alone and in combination with clomiphene citrate in polycystic ovarian syndrome in patients with insulin resistance was also confirmed by Kamenov et al. [21]. Whether the addition of melatonin will represent a benefit must be confirmed by more studies but first data suggest this [22].

This confirms that myoinositol is not only an effective alternative in the treatment of PCOS patients but also a secure one as no side effects could be observed in the standard

dosage. This is on the other side relevant as the compliance of the use rises resulting in better outcomes in the management of ovulation, hyperandrogenism, and metabolic parameters on patients with a PCOS.

Appendix

German Questionnaire

Questionnaire Number

Birth date of the patient: Month/Year
Intake of Clavella because of Sterility

(1) Sterility: primary - secondary

(2) Since when is sterility known? (Number of months):

(3) Causes for sterility: female - female and male - idiopathic

(4) Functional:

Cyclical disorders (amenorrhea; oligomenorrhea; anovulation)
PCOS
Endometriosis
Other

(5) Non-functional:

Tubal disorders
Immunological disorders
Adhesions
Other

(6) Sterility treatment in the medical history

Clomiphene
Stimulation with gonadotropins
IvF/ICSI
Other

(7) How long treatment with Clavella was performed? Months:

(8) Additional treatment? Yes - No

(9) If yes: which treatment?

(10) Did a pregnancy occur?

(11) If yes; how long after starting treatment?

Competing Interests

Pedro-Antonio Regidor is medical director of the company Exeltis GmbH, Germany, a member of the Chemo Group, which distributes myoinositol in Germany.

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Clinical Study

Prospective Randomized Study on the Influence of Myoinositol in PCOS Women Undergoing IVF in the Improvement of Oocyte Quality, Fertilization Rate, and Embryo Quality

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Polycystic ovarian syndrome (PCOS) is one of the pathological factors involved in the failure of in vitro fertilization (IvF). The aim of the present study was to investigate if the combination of myoinositol + folic acid was able to improve the oocyte quality, the ratio between follicles and retrieved oocytes, the fertilization rate, and the embryo quality in PCOS patients undergoing IvF treatments. 29 patients with PCOS underwent IvF protocols for infertility treatment and were randomized prospectively into two groups. Group A (placebo) with 15 patients and group B (4000 mg myoinositol + 400 μg folic acid per day) with 14 patients. The patients of group B used for two months myoinositol + folic acid before starting the IvF protocol and data were obtained concerning number of follicles, number of oocytes, quality of oocytes, fertilization rates, and embryo quality in both groups. The ratio follicle/retrieved oocyte was better in the myoinositol group (= group B). Out of the 233 oocytes collected in the myoinositol group 136 were fertilized, whereas only 128 out of 300 oocytes in the placebo group were fertilized. More metaphase II and I oocytes were retrieved in relation to the total amount of oocytes in the myoinositol. More embryos of grade I quality were obtained in the myoinositol. The duration of stimulation was 9,7 days (±3,3) in the myoinositol group and 11,2 (±1,8) days in the placebo group and the number of used FSH units was lower in the myoinositol group: 1750 FSH units (mean) versus 1850 units (mean). Our evidence suggests that myoinositol therapy in women with PCOS results in better fertilization rates and a clear trend to a better embryo quality. As the number of retrieved oocytes was smaller in the myoinositol group, the risk of hyper stimulation syndrome can be reduced in these patients.

1. Introduction

The Polycystic Ovary Syndrome (PCOS) is a disease that causes irregular bleeding, chronic anovulation, androgen excess, and a typical ovarian ultrasound feature [1]. It is the most common cause of infertility affecting between 5% and 10% of women during their reproductive age [2]. The etiology of the syndrome is still unknown. The European Society of Human Reproduction and Embryology and the American Society for reproductive Medicine defined at a meeting in Rotterdam 2003 the criteria for the definition of this disease [3, 4]. In order to reach a general agreement on diagnostic criteria many investigations have focused, independently of the above-mentioned disorders, on the impaired glucose tolerance, which affects 30–40% of women with PCOS [5], and on insulin resistance. Insulin resistance is common in PCOS women, regardless of the body mass index (BMI). Hyperinsulinemia due to insulin resistance occurs in up to 80% of women with PCOS and central obesity as well as in 30–40% of lean women diagnosed with this syndrome [6, 7].

Studies have suggested that an impairment of the insulin pathway could be due to a defect in the inositol phosphoglycans (IPGs) second messenger [8, 9]. IPGs play a role in activating enzymes that control glucose metabolism [10, 11]. In PCOS women, a defect in tissue availability or altered metabolism of inositol or IPGs mediators may contribute to insulin resistance [12]. Previous studies have demonstrated that myoinositol is able to restore spontaneous ovarian activity in PCOS women and consequently fertility in many of these cases [13]. Many of these PCOS women require

techniques of assisted reproduction to achieve a pregnancy. However, more than 60% of in vitro fertilization (IVF) cycles do not result in a pregnancy and poor oocyte quality is the main cause of fertilization failure in assisted reproductive techniques [14]. Therefore assisted reproductive techniques nowadays focus on obtaining high quality oocytes rather than high numbers of oocytes and embryos [15]. In IVF techniques, the supplementation with myoinositol is positively correlated to a meiotic progression of mouse germinal vesicle oocytes, enhancing intracellular $Ca2^{+}$ oscillations [16]. In follicular human fluids, higher concentrations of myoinositol represent a marker of good-quality oocytes [17].

Whether at the end myoinositol or D-Chiro-Inositol has a better effect on the quality of the oocytes in IVF cycles has to be elucidated. The paradox theory published and discussed by Nestler and Carlomagno [18, 19] suggests that D-Chiro Inositol in not physiological high dosages has a negative impact on the quality of oocytes of PCOS women, as the ovarian epimerase function is not altered in PCOS patients so that the myoinositol levels in the follicular fluids remain low if only D-Chiro-Inositol is supplemented. The Consensus Conference on myoinositol and D-Chiro-Inositol postulated that the future ideal inositol supplementation should therefore contain a ratio of 40 : 1 between myoinositol and D-Chiro-Inositol [20].

The approach of myoinositol with 3 mg melatonin is also a way in the possible improvement of oocyte quality. Rizzo et al. [21] and Unfer et al. [22] could obtain oocytes with a better quality when the PCOS women were treated in combination with myoinositol 4 grams + 400 μg folic acid + 3 mg melatonin per day in comparison to formulations without melatonin.

The aim of the present study was to investigate if pretreatment with only myoinositol and folic acid as a food supplement was able to improve the oocyte quality, the ratio between follicles and retrieved oocytes, the fertilization rate, and the embryo quality in PCOS patients undergoing IVF treatments. Due to the paradox theory, D-Chiro-Inositol was not used and melatonin 3 mg per day was excluded because of its drug status in Germany.

2. Materials and Methods

2.1. Patients. Twenty-nine patients with PCOS underwent IVF protocols for infertility treatment in the Centre for Reproductive Medicine Bogenhausen in Munich, Germany, and were randomized prospectively into two groups. Group A (placebo) with 15 patients and group B (4000 mg myoinositol + 400 μg folic acid per day) with 14 patients were evaluated. The patients of group B used for two months a combination of myoinositol and folic acid before starting the IVF protocol. The patients aged < 40 with PCOS indicated by oligomenorrhoea and/or hyperandrogenism and/or hyperandrogenemia and/or typical features of ovaries on ultrasound scan were enrolled in this study. At least two of the above-mentioned criteria were present in all the patients. The women had no other medical conditions causing ovulatory disorders such as hyperprolactinemia or thyroidal disorders or Cushing syndrome.

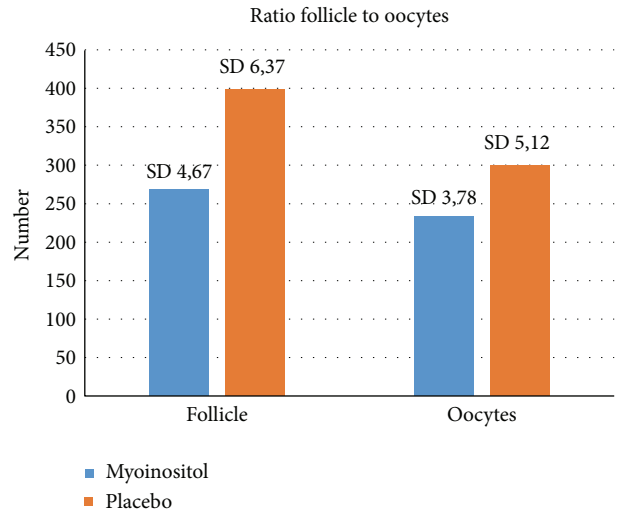

Figure 1: Ratio of follicles and retrieved oocytes. Statistically differences ($p < 0.05$) were observed. The amount of developed follicles was lower in the myoinositol group and in relation to this a higher number of oocytes were retrieved.

2.2. Controlled Ovarian Hyperstimulation. In all patients, the stimulation was started on cycle day 2 or 3 with a single dose of 150 IU gonadotropin ß. All patients received from the 6th stimulation day the antagonist Orgalutran® (Ganirelix) to prevent a premature ovulation.

Ovulation induction was performed on all the patients on day 10 or 11 of the stimulation either with 5000 IU Brevactid® (hCG), or in case of risk of an overhyperstimulation syndrome with 0.2 mg Decapeptyl® (GnRH-agonist).

Follicular puncture was performed on all patients exactly 35 hours after induction of ovulation. Those patients at risk of an overstimulation syndrome did not received an embryo transfer but the fertilized oocytes were cryoconserved at the preembryonic stage. Embryo transfer (maximum 2 embryos) was performed after 2, 3, or 5 days. Pregnancy tests were performed always 14 days after follicular puncture.

2.3. Luteal Phase. Vaginal administration of 400 mg micronized progesterone was started on the day of ovum pick-up and treatment was continued until either a serum pregnancy test result was negative or an embryonic heart was sonographically confirmed.

Data were obtained concerning number of follicles, number of retrieved oocytes, ratio follicles/oocytes, quality of oocytes, fertilization rates, and embryo quality.

For statistically analyses a Student's t-test was performed to compare the effects between the treatment groups.

3. Results

Group A (= placebo group) showed a higher amount of retrieved oocytes than group B. Nevertheless the ratio follicle/retrieved oocyte was clearly lower in the myoinositol group (= group B) ($p < 0.05$) (see Figure 1).

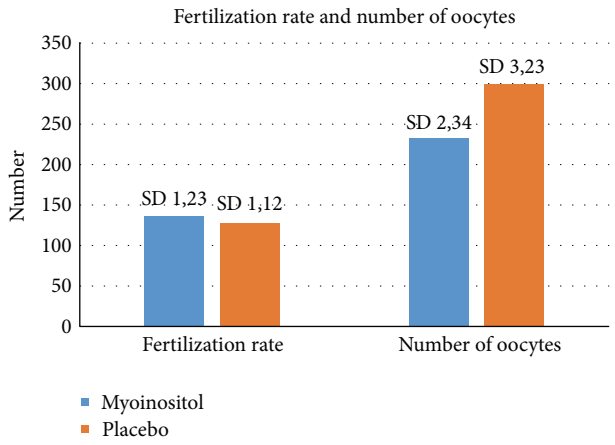

Figure 2: Relation between the myoinositol treatment and the fertilization rate in both groups. Higher rates ($p < 0.05$) were observed in the myoinositol group in comparison to the placebo group.

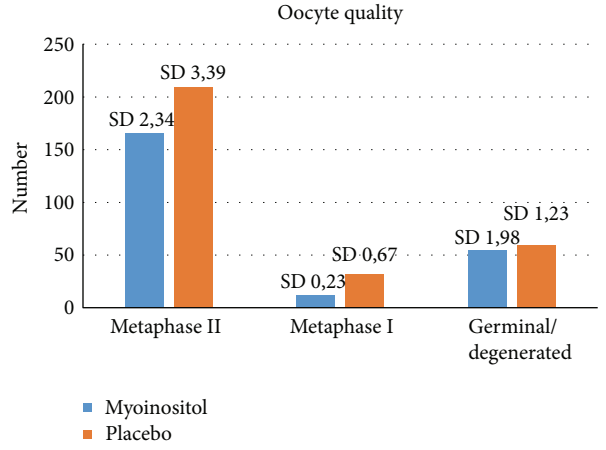

Figure 3: Oocyte quality and treatment with myoinositol or placebo ($p = 0.06$).

From the obtained 233 oocytes in the myoinositol group 136 were fertilized whereas only 128 of 300 oocytes were fertilized in the placebo group ($p < 0.05$) (see Figure 2).

In relation to the quality of the oocytes, better data were obtained in the myoinositol group. More metaphase II and I oocytes were retrieved in relation to the total amount of oocytes when compared with the placebo group ($p > 0.05$) (see Figure 3).

The last analysis that was performed on the quality of embryos. More embryos of grade I quality were observed in the myoinositol group than in the placebo group ($p < 0.05$) (Figure 4). This difference was, as the analyses done for the fertilization rate and the ratio follicles retrieved oocytes, statistically significant.

The amount of used FSH units was lower in the group of women that received myoinositol. A mean of 1750 units was used (minimum 1350 units and maximum 2250 units whereas the used amount in the placebo group was higher. Mean used units: 1850 with a minimum 1500 and a maximum of 2300

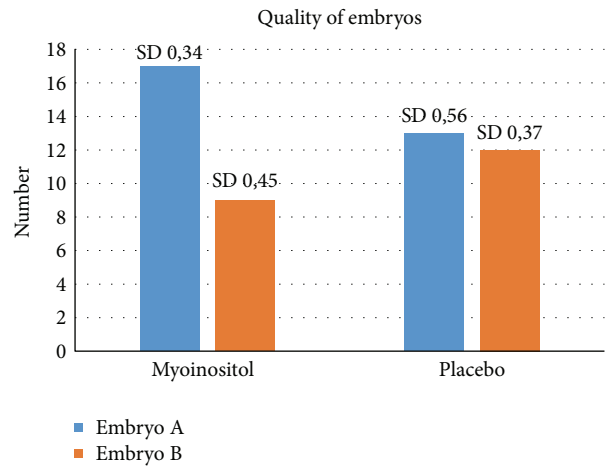

Figure 4: Embryo quality after treatment with myoinositol or placebo. Higher number of embryos with grade I were observed in the myoinositol group ($p < 0.05$).

units ($p > 0.05$)). In regard to the amount of stimulation days a significant difference was observed. The myoinositol patients were stimulated with FSH in the mean 9,7 days (±3,3 days) and the placebo group 11,2 days (±1,8 days). This difference was significant ($p < 0.05$).

4. Discussion

The Polycystic Ovary Syndrome is one of the most common endocrine disorders affecting women. Insulin resistance and hyperinsulinemia are often found in a high proportion of women with PCOS. A defect in insulin action has been postulated, possibly as a consequence of a deficiency of D-Chiro-Inositol, which is a component of inositol phosphoglycans. Insulin-lowering medications, particularly myoinositol, represent novel therapies for restoring spontaneous ovulation, with a potential positive effect also on human oocyte meiotic maturation. This therapy appears to influence steroidogenesis directly, reducing the androgen production in theca cells. It was shown that inositol administration increases the action of insulin in patients with PCOS, thereby improving ovulatory function and decreasing serum testosterone concentration [12, 23–26].

Studies have shown the positive effects of myoinositol on the outcome of IVF cycles in women with a history of a PCOS. Papaleo et al. [13] could show that in patients with PCOS the treatment with myoinositol and folic acid, but not folic acid alone, reduces germinal vesicles and degenerated oocytes at ovum pick-up without compromising total number of retrieved oocytes. By the same way the amount of stimulation days was, as in our study, shorter in the myoinositol treated women showing a faster response of the ovarian follicles to FSH stimulation due to the myoinositol effects.

On the other side PCOS women have an increased risk of hyper stimulation syndrome [27]. High levels of serum ovarian androgens are associated to a production of elevated serum E2 levels after gonadotropin ovarian stimulation. The study of Papaleo et al. (2009) showed that PCOS patients

treated with myoinositol and gonadotropin had a significant reduction in E2 levels after hGC administration [28]. This was related also to the lower number of in vitro fertilization (IVF) cycles cancelled because of high E2 levels (sign of hyper stimulation syndrome).

In addition to this, myoinositol is an important constituent of follicular fluid, playing a key role in both nuclear and cytoplasmic oocyte development. In fact, supplementation with myoinositol in the IVF technique is positively associated to meiotic progression of mouse germinal vesicle oocytes, enhancing intracellular Ca^{2+} oscillation [17]. In human follicular fluids, higher concentrations of MI provide a marker of good-quality oocytes [16, 28, 29].

Beside the increased ratio follicles/retrieved oocytes and the higher fertilization rate in the group of women that used myoinositol, a more important result was the increased number of top-quality (score 1 versus 2) embryos in the myoinositol group. These data suggest that, besides increasing the fertilization rate, myoinositol supplementation has also an effect on the overall quality of the oocyte pool.

Though, additional investigations on larger number of patients are needed to further characterize the impact of myoinositol treatment on oocyte follicular development and oocyte maturation and its implication in stimulation and pregnancy outcomes in IVF procedures.

Competing Interests

Dr. Bernd Lesoine has no competing interests. Dr. Pedro-Antonio Regidor is employee of Exeltis Germany.

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Clinical Study

Myo-Inositol in the Treatment of Teenagers Affected by PCOS

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Objective. To compare the effectiveness of myo-inositol (MI) and oral contraceptive pills (OCPs) in monotherapy and MI in combination with OCPs in the treatment of teenagers affected by polycystic ovary syndrome (PCOS). *Methods*. 61 adolescent girls aged 13–19 years, with PCOS, were involved in the prospective, open-label study. Patients were randomized into three groups: I group, 20 patients receiving drospirenone 3 mg/ethinyl estradiol 30 μg; II group, 20 patients receiving 4 g myo-inositol plus 400 mg folic acid; III group, 21 patients receiving both medications. *Results*. After receiving MI significant reduction in weight, BMI, glucose, C-peptide, insulin, HOMA-IR, FT, and LH was detected. The levels of SHBG, TT, FAI, DHEA-S, and AMH did not change statistically significantly. After receiving OCPs weight and BMI slightly increased, but metabolic parameters did not change. Combination of MI and OCPs did not change weight and BMI, but reduction in C-peptide, insulin, and HOMA-IR was detected. TT, FT, FAI, DHEA-S, LH, and AMH levels decreased and SHBG increased. *Conclusions*. Administration of MI is a safe and effective method to prevent and correct metabolic disorders in teenagers affected by PCOS. With combination of MI and OCPs antiandrogenic effects are enhanced, negative impact of OCPs on weight gain is balanced, and metabolic profile is improved.

1. Introduction

Polycystic ovary syndrome (PCOS) is considered to be the most common endocrine disorder in the women of reproductive age, with onset manifesting as early as puberty [1]. PCOS prevalence is estimated to be about 6–8%, although with the implementation of the Rotterdam criteria, the prevalence increased up to 15–25%, while the use of AES recommendations put PCOS prevalence at about 10–15% [2].

PCOS is a genetically determined lifelong disease. Symptoms start from the early prepubertal years and continue after menopause. The phenotypic expression varies through time, depending on several internal and external factors [3, 4].

The data suggests that exposure of a female to harmful events during fetal life and the peripubertal period may considerably affect her metabolic, hormonal, and reproductive phenotype. Dysregulation of cytochrome P450 p17, the androgen-forming enzyme in both adrenal glands and the ovaries, is the central pathogenic mechanism underlying hyperandrogenism in PCOS [5]. The presence of hyperandrogenism reduces the hepatic synthesis of SHBG and leads to a relative excess of free circulating androgens [6].

Adolescents with PCOS tend to be troubled by the cosmetic effects, such as acne, hirsutism, acanthosis nigricans, and obesity. They occur during a particularly vulnerable stage of their psychological development [7].

Insulin resistance and compensatory hyperinsulinemia are observable in at least 45–65% of PCOS patients and frequently appear to be related to excessive serine phosphorylation of the insulin receptor [5].

Patients with PCOS have an increased risk for developing type 2 diabetes, metabolic syndrome, coronary heart disease, and endometrial cancer [8, 9]. Obese PCOS adolescents are at increased risk for developing glucose intolerance and type 2 diabetes compared with their non-PCOS peers [10–12].

The preferred effective method of treatment for obese adolescents with PCOS is lifestyle modification; however it is hard for patients to comply with and achieve this [13].

Combined oral contraceptive pills (OCPs) have been used in the women with PCOS for treatment of menstrual disorders, acne, and hirsutism. Despite years of using them and broad clinical experience, there still are some ongoing doubts concerning their implications for the cardiovascular system and carbohydrate metabolism [14]. Most pediatric

endocrinologists prefer to use OCPs with low androgenic potential [15].

More recently, insulin-sensitizing drugs have been proposed as another long-term treatment for PCOS. Pharmacologic reduction in insulin levels by either metformin or thiazolidinediones ameliorates both hyperinsulinemia and hyperandrogenism [16, 17]. A meta-analysis of the published studies demonstrated that the use of insulin sensitizers does not reduce hyperandrogenism any better than OCPs [18].

Adolescent girls with PCOS require a long-term committed treatment that can have serious side effects. Therefore, alternative treatments may be possible options for adolescents affected by PCOS [7].

In the last decade, a higher attention has been paid to the role of inositol-phosphoglycan (IPC) mediators of insulin action [19]. A deficiency of myo-inositol (MI) has been found in women with PCOS affected by insulin resistance [20]. The insulin resistance at the base of PCOS leads to hyperinsulinemia, which at ovarian level is responsible for the alteration in the inositols metabolism. PCOS patients with hyperinsulinemia present an enhanced MI to D-chiro-inositol (DCI) epimerization in the ovary; this results in an increased DCI/MI ratio (i.e., overproduction of DCI). A sequent reduction of MI increases the isoform DCI and induces overproduction of androgens [21–23]. MI is a component of the vitamin B complex and insulin sensitizer, which improves insulin signaling, reduces serum insulin, and decreases serum testosterone, thereby restoring normal ovulatory function in PCOS women [24–27].

Safe and effective treatment in teenagers affected by PCOS is essential for ameliorating clinical manifestations, restoring self-esteem, and preventing further complications. There are limited studies concerning usage of MI in adolescents. Therefore, the aim of our study was to compare the effectiveness of MI and OCPs in monotherapy and combination of MI and OCPs in the treatment of teenagers with PCOS by evaluation of hormonal and metabolic profile.

2. Materials and Methods

61 adolescent girls aged 13–19 years affected by PCOS were involved in the prospective, open-label study. The diagnosis was confirmed according to the Rotterdam criteria [28]. Patients within two years of menarche were excluded from the study. All the patients were from Archil Khomasuridze Institute of Reproductology. Informed written consent was obtained from all participants and/or their mothers (guardians) before entering the trial and the local committee of ethics approved the given study.

Healthy life style, including reduced carbohydrate intake and gentle exercise, was recommended to all patients as a 1st line nonpharmacological management, especially to overweight ones. Patients were randomly allocated into three following treatment groups: I group, 20 patients receiving monophasic low-dose combined OCPs, Yarina (drospirenone 3 mg/ethinyl estradiol 30 μg, Bayer Health Care Pharmaceuticals), consumed in the evening in a cyclic regimen (from the 3rd to the 5th day of menstruation, 21 days) for 3 months; II group, 20 patients receiving Inofolic (2 g myo-inositol plus 200 mg folic acid, Lo.Li., Pharma S.r.l., Rome, Italy) consuming 2 g in the morning and 2 g in the evening for 3 months; III group, 21 patients receiving combination of Yarina and Inofolic in the same regimen for 3 months.

Evaluations of anthropometric, endocrine, and metabolic parameters were conducted before and after 3 months of treatment. Body mass index (BMI) was calculated as weight (kg) divided by height (m^2).

Serum concentrations of prolactin (PRL), luteinizing hormone (LH), total testosterone (TT), sex hormone-binding globulin (SHBG), free-testosterone (FT), dehydroepiandrosterone-sulfate (DHEA-S), anti-Mullerian hormone (AMH), C-peptide, fasting glucose, and fasting insulin levels were assessed within the first 5 days of the menstrual cycle. Insulin resistance was measured by homeostasis model assessment (HOMA-IR) and free androgen index (FAI) was calculated by formula: ((TT nmol/l)/(SHBG nmol/l)) × 100.

Statistical analysis was performed by using SPSS (Statistical Package for Social Sciences, version 21, Chicago, USA). Data are given as mean ± SD. The baseline characteristics of the patients between groups and changes in parameters within groups after 3 months of treatment were assessed using unpaired t-test (ANOVA). The results were considered as statistically significant when the p value was less than 0.05 ($p < 0.05$).

3. Results

In the I group (Yarina) average age of the patients was 15.95 ± 1.85, average weight was 61.1 ± 9.92 kg, and average BMI was 22.74 ± 3.75 kg/m^2. Among them 5 patients (25%) had BMI > 25.5 kg/m^2. In the II group (Inofolic) average age of the patients was 16.75 ± 2.0, average weight was 58.6 ± 9.3 kg, and average BMI was 22.3±3.08 kg/m^2 and among them 4 patients (20%) had BMI > 25.5 kg/m^2. In the III group (Yarina plus Inofolic) average age of the patients was 16.24 ± 1.86, average weight was 58.95 ± 9.6, and average BMI was 22.24 ± 3.26. Among these patients 4 (19%) had BMI > 25.5 kg/m^2.

Hormonal and metabolic parameters of patients are listed in Table 1. Groups were well matched at baseline—there were no statistically significant differences between the groups in terms of clinical, anthropometric, hormonal, and metabolic parameters. Average LH level only was higher in III group compared with other groups ($p < 0.05$).

In the I group after receiving Yarina, patient's weight and BMI increased statistically significantly, while in the II group after receiving Inofolic, patient's weight and BMI decreased statistically significantly. In the III group, in patients treated with combination of Yarina and Inofolic, we did not reveal statistically significant changes in average weight and BMI.

The average level of PRL did not change before and after treatment in groups. The average level of LH was statistically significantly decreased after treatment in all groups, but less significantly in patients treated with Inofolic. The average level of DHEA-S did not change in patients after receiving Inofolic but decreased statistically significantly in the Yarina and combined groups.

Table 1: Anthropometric, endocrine, and metabolic parameters of the patients in treatment groups.

Parameter M ± SD	Yarina Group I		Inofolic Group II		Yarina + inofolic Group III	
	Baseline	After 3 months	Baseline	After 3 months	Baseline	After 3 months
BMI kg/m^2	22.74 ± 3.75	23.03 ± 3.6*	22.3 ± 3.08	21.9 ± 2.5*	22.24 ± 3.26	21.99 ± 2.57
Weight kg	61.1 ± 9.92	61.9 ± 9.47*	58.6 ± 9.3	57.4 ± 7.6*	58.95 ± 9.6	58.05 ± 7.6
PRL ng/ml	11.70 ± 5.11	12.01 ± 4.5	12.98 ± 5.75	12.5 ± 5.32	13.39 ± 4.5	13.03 ± 4.35
LH IU/I	10.42 ± 3.9	8.01 ± 2.9**	9.11 ± 5.7	7.56 ± 4.5*	13.1 ± 5.58	8.42 ± 3.7**
DHEA-S μg/ml	2.03 ± 0.7	1.82 ± 0.46*	2.09 ± 0.50	1.89 ± 0.59	2.3 ± 0.67	1.9 ± 0.38*
TT ng/ml	0.72 ± 0.25	0.58 ± 0.57	0.73 ± 0.25	0.73 ± 0.46	0.70 ± 0.23	0.51 ± 0.16**
FT pg/ml	4.26 ± 2.46	2.74 ± 1.16*	3.82 ± 2.01	2.98 ± 0.94*	3.42 ± 2.16	2.12 ± 0.8**
FAI	7.13 ± 3.8	3.8 ± 2.6**	6.7 ± 5.7	5.99 ± 5.24	8.1 ± 5.5	2.94 ± 1.04**
SHBG nmol/l	45.03 ± 24.39	55.42 ± 25.7**	53.7 ± 27.4	56.29 ± 24.3	47.4 ± 29.2	65.01 ± 22.5**
Glucose mmol/l	4.61 ± 0.74	4.36 ± 0.53*	4.64 ± 0.65	4.39 ± 0.56*	4.64 ± 0.72	4.42 ± 0.52
C-peptide ng/ml	1.6 ± 0.7	1.53 ± 0.34	1.92 ± 0.7	1.82 ± 0.34*	1.52 ± 0.7	1.22 ± 0.57*
Insulin μIU/ml	8.07 ± 5.24	8.12 ± 4.3	8.5 ± 6.7	5.2 ± 3.04*	8.67 ± 4.7	6.67 ± 2.79*
HOMA-IR	1.59 ± 1.07	1.57 ± 0.87	1.81 ± 1.38	1.03 ± 0.64*	1.73 ± 1.0	1.3 ± 0.56*
AMH ng/ml	11.72 ± 5.8	10.26 ± 4.6**	11.5 ± 5.8	12.6 ± 6.25	12.01 ± 5.6	10.4 ± 4.7*

Comparison of the data at baseline and after treatment within the groups. $^*p < 0.05$; $^{**}p < 0.001$.

Reduction in TT was observed in the I and III groups, but statistically significantly, only in the III group. The change was not revealed after receiving Inofolic only. However, average FT decreased statistically significantly in all groups and more significantly in the combined group. Average SHBG level was increased in all treatment groups, but statistically significantly only in the I and III groups. Accordingly, FAI was reduced significantly in the Yarina and combined groups and change was not detected in the Inofolic group.

The average level of AMH statistically significantly decreased in patients receiving Yarina only and combination of Yarina and Inofolic, but more significantly in I group. But in the II group, after treatment of Inofolic only, it increased, but not statistically significantly.

Before treatment all patients had normal levels of fasting glucose, but hyperinsulinemia and insulin resistance were observed in 6 (30%) and 5 (25%) patients in I group, in 6 (30%) and 4 (20%) patients in II group, and in 6 (29%) and 5 (24%) patients in III group, respectively.

Statistically significant reduction in average of glucose, C-peptide, insulin concentration, and HOMA-IR was detected in patients after receiving Inofolic. However, in the patients receiving Yarina only statistically significant changes were not revealed in average concentrations of C-peptide, insulin, and HOMA-IR. However, the average levels of glucose decreased statistically significantly. All these parameters significantly decreased in patients treated with combination of Yarina and Inofolic, except for the glucose concentration; the change was not statistically significant.

4. Discussion

PCOS is quite a complex syndrome and should not be considered as "an easy to treat" disease. It needs a precise clinical screening that might give suggestions on what hormonal and metabolic parameters need to be treated [29]. This issue is extremely important in adolescents, due to the diagnostic difficulties in this period.

In the first year after menarche half of the cycles are anovulatory in healthy adolescents. If menstrual irregularities persist 2 years after menarche the risk of PCOS is extremely high, 70% of cases [30].

Generally, the first line nonpharmacological management of PCOS includes life style modification with loss of weight. It has been proven as an effective way for restoring ovulatory cycles and achieving pregnancy in overweight women with PCOS [31]. Therefore, healthy life style modification was recommended to all our patients, with reduced carbohydrate intake and gentle exercising to those who were overweight.

Long-term management of PCOS most often involves the use of OCPs [32]. OCPs effectively reduce hirsutism and acne in women with PCOS. This improvement is usually revealed in 60–100% of cases after at least 6 months of treatment [33]. The effect of OCPs on hyperandrogenism includes suppression of androgen production in the ovary by inhibiting secretion of LH, induction of SHBG synthesis in the liver and consequent decrease in FT, slight decrease in adrenal androgen production, and direct antiandrogenic effect of a progestin component of OCPS [34]. According to the results of our study after receiving Yarina TT decreased, but not statistically significantly, which can be explained by small size of the group. However, increase in SHBG and decrease in FAI and FT were detected. DHEA-S decreased as well. We did not reveal reduction in androgens after treatment with MI in our study in contrast to others [35]. It can be related to limited period of our observation. However, after treatment with MI decrease of LH level was detected, which is a precondition for antiandrogenic activity and is reported by other authors

as well [35]. It should be emphasized that after receiving MI the amount of FT is reduced.

With combination of MI and OCPs antiandrogenic activity was extremely marked and was expressed by significant reduction in TT, FT, DHEA-S, FAI, and LH.

Recent studies demonstrate effectiveness of MI in the treatment of hirsutism and other cutaneous disorders in young women with PCOS [35, 36]. Due to the short duration of our study, we did not evaluate the expression of clinical manifestations, like hirsutism, acne, acanthosis nigricans, and changes in menstrual cycle, but positive effect of all medications on hormonal profile is apparent.

The serum AMH level strongly correlates with the number of small antral follicles and is closely related to the degree of menstrual disturbances [37]. Therefore high AMH levels were observed in patients with PCOS. This is particularly useful for the diagnosis of PCO [38, 39]. In our study baseline level of AMH was elevated almost in all patients. After treatment with OCPs only and in combination with MI, AMH level decreased, while after treatment with MI only trend to increase AMH was detected.

MI improves response to clomiphene citrate in infertile women, restores ovulation, and increases clinical pregnancy and live birth rate [40]. Latest studies proved effectiveness of MI in improving quality of oocytes in IVF cycles [41]. Therefore, these beneficial effects of MI would be helpful in adolescents with PCOS to prevent reproductive disorders in future.

Typically, after receiving OCPs weight increases. In drospirenone containing pills, this effect is less expressed [42]. In our study after receiving Yarina average weight and BMI slightly increased. It could be related to increase of appetite.

We consider weight loss and BMI decreasing to be quite pivotal after receiving MI. In the study of Gerli et al. a significant weight loss in patients treated with MI was recorded [43]. In other studies changes in BMI of reproductive age women were not detected [35]. We underline that in our study in patients treated with combination of MI and OCPs average weight and BMI did not change. Apparently, MI balances negative impact of OCPs.

The studies conducted in obese women with PCOS demonstrated a deterioration of glucose tolerance with OCPs administration likely due to a decrease in insulin sensitivity with no change in plasma insulin concentrations [44, 45]. This occurred despite no change in BMI and a marked decrease in circulating androgens. Studies performed in nonobese women with PCOS showed no change in glucose tolerance or insulin sensitivity after OCPs, suggesting that the metabolic effects of OCPs may vary with body phenotype [46, 47]. During usage of drospirenone the metabolic effects appear to be much less severe or entirely nonexistent when women with PCOS are treated with drospirenone containing OCPs. Guido and authors found no significant change in insulin sensitivity in PCOS women treated with drospirenone containing OCPs [48]. The use of drospirenone appears to alleviate the metabolic concerns that are specific for women with PCOS [15].

In our study MI showed good results in terms of controlling metabolic parameters, glucose, C-peptide, insulin, and HOMA-IR, and demonstrated slight antiandrogenic activity, which is reported by other authors as well [43]. According to our results, changes in metabolic parameters matched with weight reduction. It is very important to underline that during receiving Yarina metabolic profile did not change, but in combination of MI, it improved. It is considerable finding that MI can prevent developing of serious metabolic disturbances in adolescents with PCOS in future.

According to the literature, minimal weight loss of 2–7% of body weight reduces androgen levels and improves ovulatory function in many patients with PCOS [49]. In adolescents lifestyle modification can only result in a 59% reduction of free androgen index with a 122% increase in SHBG [50]. So, it is difficult to say how the modification of lifestyle and administration of medications contributed to reduction of androgens and controlling metabolic parameters in our study.

5. Conclusion

Administration of MI is a safe and effective method to prevent and correct metabolic disorders in teenagers affected by PCOS. With combination of MI and OCPs antiandrogenic effects are enhanced, negative impact of OCPs on weight gain is balanced, and metabolic profile is improved.

A simultaneous treatment with MI and OCPs (containing drospirenone) on the basis of life-style modification can be considered as a highly effective approach in teenagers affected by PCOS.

Competing Interests

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

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Review Article

Metabolism and Ovarian Function in PCOS Women: A Therapeutic Approach with Inositols

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Polycystic ovary syndrome (PCOS) is characterized by chronical anovulation and hyperandrogenism which may be present in a different degree of severity. Insulin-resistance and hyperinsulinemia are the main physiopathological basis of this syndrome and the failure of inositol-mediated signaling may concur to them. Myo (MI) and D-chiro-inositol (DCI), the most studied inositol isoforms, are classified as insulin sensitizers. In form of glycans, DCI-phosphoglycan and MI-phosphoglycan control key enzymes were involved in glucose and lipid metabolism. In form of phosphoinositides, they play an important role as second messengers in several cellular biological functions. Considering the key role played by insulin-resistance and androgen excess in PCOS patients, the insulin-sensitizing effects of both MI and DCI were tested in order to ameliorate symptoms and signs of this syndrome, including the possibility to restore patients' fertility. Accumulating evidence suggests that both isoforms of inositol are effective in improving ovarian function and metabolism in patients with PCOS, although MI showed the most marked effect on the metabolic profile, whereas DCI reduced hyperandrogenism better. The purpose of this review is to provide an update on inositol signaling and correlate data on biological functions of these multifaceted molecules, in view of a rational use for the therapy in women with PCOS.

1. PCOS Definition(s): The More, the Better?

The polycystic ovary syndrome (PCOS) is a clinical syndrome characterized by chronic anovulation and hyperandrogenism [1], affecting 6–10% [2] of women in reproductive age. Its etiology is complex, heterogeneous, and not completely understood. Current evidence suggests that genetic, endocrine, metabolic, and environmental aspects are involved in determining the syndrome.

There are three recognized definitions to diagnose PCOS, among which the Rotterdam Criteria proposed in 2003 by European Society of Human Reproduction and Embryology/American Society for the Reproductive Medicine (ESHRE/ASRM) are the most recommended [1, 2]. The Rotterdam Criteria consider at least two of three criteria between clinical or biochemical hyperandrogenism, menstrual irregularity, and polycystic ovaries characterized by ultrasound detection of 12 or more follicles <9 mm in diameter and/or increased ovarian volume >10 mL and in absence of a dominant follicle [3]. In 1990 the National Institutes of Health (NIH) proposed that PCOS might be diagnosed with hyperandrogenism and irregular menstrual cycles without knowledge of ovarian ultrasound pattern [4]. Instead, the Androgen Excess PCOS Society [5] recommends diagnosis in the presence of hyperandrogenism plus one of two other criteria among ovulation dysfunction and PCO morphology, according to Rotterdam Criteria (Table 1). Nevertheless, all

Table 1: Diagnostic criteria of PCOS according to different definitions.

	NIH (1990) necessary 2 criteria	Rotterdam criteria (2003) necessary at least 2 criteria	Androgen Excess PCOS Society (2009) necessary at least 2 criteria
Clinical hyperandrogenism or biochemical hyperandrogenism	Obligatory presence	Possible presence	Obligatory presence
Oligo/anovulation may manifest with frequent or infrequent bleeding, respectively, at <21 days or >35 days Rarely cycles may be anovulatory despite regular bleeding	Obligatory presence	Possible presence	Possible presence
Ultrasound polycystic ovarian features: presence of 12 or more follicles 2–9 mm in diameter and/or increased ovarian volume >10 mL (without dominant follicle) in either ovary	—	Possible presence	Possible presence

three definitions underline the importance to exclude other clinical disorders that may mimic PCOS.

Many aspects of this syndrome are still debated; for example, which criteria may be adopted to make a diagnosis during adolescence? The third PCOS consensus workshop in 2010 identified gaps in knowledge and dealt with these controversial aspects [6]. During adolescence as many as 85% of menstrual cycles are anovulatory during the first years following menarche and as many as 59% are anovulatory up to three years after the menarche in physiological conditions [7]. Furthermore only 40% of adolescents with irregular cycles display polycystic ovary on ultrasound [8]. On the other hand, other authors found in adolescent population a high prevalence of characteristic polycystic ovaries in otherwise asymptomatic girls, suggesting a high prevalence of this feature in this age range [9]. In addition, hyperandrogenism seems a most important feature to lead diagnosis during adolescence [10]. Basing on these data, it was suggested that all three criteria should be present to diagnose PCOS in adolescents and that irregularity in menstrual cycles must persist for at least 2 years after menarche [11].

2. PCOS and Metabolic Aspects: The Key Role of Insulin-Resistance

Insulin-resistance and hyperinsulinemia are tightly related to pathogenesis of PCOS [12, 13] which may be exacerbated by coexistence of obesity, affecting about 50% of women with PCOS [14]. Nevertheless, many studies showed that insulin-resistance affected also normal weight women with PCOS [15, 16]. Besides, in lean patients with PCOS the visceral adiposity seems to be greater than in nonobese women without PCOS and this feature might in part explain metabolic disorders in these patients [16].

Overall between 50 and 70% of women with PCOS had demonstrable insulin-resistance [5], which results in a compensatory increase in insulin secretion by β-islet cells of the pancreas. However, many women with insulin-resistance and PCOS also had impaired β cell function and the grade of dysfunction seems to be related to family history of type II diabetes [17]. Furthermore, hyperinsulinemic insulin-resistance stimulates the ovarian androgen production and increases the likelihood to develop type 2 diabetes, metabolic syndrome, and cardiovascular disease [18].

About 22% of women under 20 years and 53% between 30 and 39 years of age affected by PCOS met the criteria for metabolic syndrome (MS) according to the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) [19]. Indeed, the prevalence of MS in patients with PCOS seems to be 2-fold higher than reported for age-matched general population even when stratified by both age and Body Mass Index (BMI). Basing on these epidemiological data, it is possible that the presence of PCOS by itself conferred an increased risk of MS, since the intrinsic insulin-resistance of PCOS patients [20].

Other authors [21] found a higher risk of MS in young patients with PCOS compared with general age-matched population such as 37% versus 5% and 47 versus 13%, according to two different criteria proposed for diagnosis of MS [22, 23]; furthermore among obese young patients the prevalence increased to 63%. Obesity and insulin-resistance were significant risk factors to develop MS, but hyperandrogenemia remained a significant predictor of MS after adjusting for both obesity and insulin-resistance: in fact the prevalence of MS was higher in patients with PCOS and higher bioavailable testosterone levels [21]. The prevalence of type 2 diabetes in women with PCOS was 8%, 8-fold greater than general population, and prevalence of impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) was 11% and 0.9%, respectively. According to the 2003 guidelines of the Expert Committee on the diagnosis and classification of diabetes mellitus [24], which established for IFG diagnosis fasting glucose blood levels higher than 100 mg/dL, the prevalence of IFG was 10% [20]. In others studies the prevalence of type 2 diabetes was similar, from 4 to 10% of women with PCOS [25].

Dyslipidemia is one of most frequent features in patients with PCOS. Hyperinsulinemia and insulin-resistance are related to abnormality of lipid profile, particularly to decreased levels of high density lipoprotein cholesterol (HDL-C) levels and increased levels of triglycerides and

of small dense low density lipoprotein cholesterol (LDL-C) [26]. Insulin-resistance causes a rise in free fatty acid (FFA) plasma levels due to increased synthesis from liver and increased mobilization from adipose tissue. The excess of FFA leads *per se* to insulin-resistance by inactivation of key enzymes such as pyruvate dehydrogenase (PDH) or by decreasing glucose transport activity, which may be a consequence of altered insulin signaling through decreased insulin receptor substrate-1 (IRS-1) associated PI3 kinase activity [27]. The Hoorn study, started in 1989 and finished in 1998 after a 10 years' follow-up, has already registered increased cardiovascular diseases (CVD) mortality among patients with MS, by 1.8- and 1.2-fold, respectively, in men and women, and all-causes mortality was increased by 1.5-fold in both sexes. Furthermore, patients with MS were 2.9-fold more likely to develop diabetes [28].

A pioneering study by Burghen et al. [12] identified a relationship between hyperandrogenism and hyperinsulinemia in polycystic ovary disease. Since those first studies it became evident that insulin-mediated glucose disposal, calculated with euglycemic glucose clamp, was decreased in PCOS as a result of insulin-resistance in skeletal muscle and fat tissue. Hepatic glucose output and its insulin-mediated suppression were also altered, consistent with hepatic insulin-resistance [29, 30]. Other authors showed that adolescents with PCOS and with IGT had the same insulin sensitivity compared to adolescents with normal glucose tolerance but showed higher hepatic glucose output and 50% lowering in the first phase of insulin secretion [31]. Other studies confirmed a decrease in both first and second phases of insulin response to glucose, suggesting abnormal β-cell function, in women with PCOS and abnormal glucose tolerance [17].

3. PCOS and Impaired Follicular Maturation

PCOS often develops during puberty inducing dermatological signs (hirsutism, acne, and alopecia), irregular menses, and biochemical alterations associated with high levels of testosterone, DHEA, androstenedione, and luteinizing hormone (LH) and increased LH/follicle-stimulating hormone (FSH) ratio, together with a concurrent reduction of sex hormone binding globulin (SHBG) and insulin-like growth factor (IGF) binding protein [32]. These alterations are due to insulin-resistance and hyperinsulinemia. Hyperinsulinemia stimulates pituitary LH release (thus increasing LH/FSH ratio), raises androgen production from ovarian theca cells, and decreases SHBG synthesis, leading to enhanced free testosterone levels. Recent studies showed that hyperandrogenism may also be due to local inflammatory response of ovarian theca cells to reactive oxygen species [33] or to cytokines and chemokines produced by dysfunctional adipose tissue [34]. In addition, obesity interferes with the hypothalamus-pituitary-ovary regulation system and therefore inhibits the physiological process of ovarian follicular maturation [35]. Overall, hyperandrogenism together with high levels of LH significantly disturbs the physiological process of ovarian follicular maturation and may lead to anovulatory cycles.

4. Rationale of Inositol-Based Therapeutic Approach in PCOS

Current evidence suggests that insulin-resistance and secondary hyperinsulinemia play an important role in hyperandrogenism, anovulation or irregular cycles, and metabolic alteration in both lean and obese patients with PCOS. The current therapy aims to improve insulin-resistance, to reach a reduction of compensatory hyperinsulinemia and then improve metabolic and ovulatory features in patients with PCOS. According to recent guidelines, insulin-sensitizer drugs are the first line therapy in women with metabolic abnormalities and irregular cycle with the purpose to improve fertility, whereas a lifestyle change with weight loss and physical activity is the first step in overweight and obese PCOS patients. Metformin is recommended both in patients with metabolic abnormalities (such as IGT, IFG, and type 2 diabetes) and in women who desire pregnancy, as an adjuvant therapy for infertility and to decrease the risk of ovarian hyperstimulation syndrome [36].

In the past, many researchers centered their studies on the role played by inositols as second messengers in insulin signaling transduction [37]. In the last two decades, inositols were found to have important effects on ovulation and metabolism [38]; other studies underlined the importance of myo-inositol (MI) in oocyte differentiation and inositol ability to improve fecundation with *in vitro* fertilization techniques in women with PCOS [39].

Today we know much more about the key role that MI and D-chiro-inositol (DCI) play in insulin signaling pathway and the crucial function of myo-inositol in oocyte maturation. Inositols belong to a family of nine stereoisomeric six-carbon cyclitols which includes myo, muco, neo, scyllo, and D- and L-chiro-inositol and other three cis, allo, and epi-inositol that do not exist in nature. MI is the most widely distributed in nature; the main source of MI is the diet, as it is found in a wide variety of foods such as whole wheat, seeds, and fruits. Inositol is assumed to be an essential B complex vitamin; however it is known that inositol can be synthesized in human body. The synthesis starts from glucose 6-phosphate (G6P), which is turned into inositol-3-phosphate and later dephosphorylated by inositol monophosphatase 1, yielding the free MI [40]. MI is then converted in DCI by epimerase in mammalian tissues.

Inositol plays a fundamental role in the cell in two different manners: (1) incorporated in membrane phospholipids, producing phosphoinositides upon membrane receptor stimulation [41] and (2) in form of inositolphosphoglycans (IPG) that can be located at the inner or outer side of the plasma membrane and are involved in insulin transduction signaling as second messengers [42].

Inositol phospholipids have long been known as fundamental components of cellular membranes, concentrated at the cytosolic surface, and have a crucial function in membrane integrity as well as in intracellular signaling. Phosphatidylinositol (PtdIns) is a precursor of arachidonic acid, an intermediate in prostaglandins synthesis. PtdIns is also a precursor of phosphoinositides by reversible phosphorylation of the inositol ring in different positions (3,4,5),

resulting in generation of seven phosphoinositide species [41], among which phosphatidylinositol 4P (PtdIns-4P) and phosphatidylinositol 4,5 bisphosphate (PtdIns 4,5P) are the most studied.

PtdIns-4P takes part in the regulation of cytoskeleton structure and PtdIns-4,5P is involved in regulation of linkage among actin and cytoskeleton with role in regulation of cellular motility; either have an important role in cellular and subcellular architecture [41].

PtdIns-4,5P is a key molecule in the "phosphoinositides signaling pathway" of G protein-coupled receptors (GPCRs) associated to phospholipase C, which generate the second messengers diacylglycerol (DAG) and inositol triphosphate (Ins 1,4,5-P). DAG in turn activates protein kinase C (PKC), an enzyme involved in many cell functions (proliferation, apoptosis, and differentiation), whereas Ins 1,4,5-P stimulates Ca^{2+} release from intracellular stores, activating several Ca^{2+}-binding proteins and inducing numerous related physiological effects [43]. Some authors showed that Ins 1,4,5P played an important role in physiopathology of oocytes fertility, triggering the Ca^{2+} rise and secondary activation phenomena at fertilization [44].

In the last thirty years, MI and DCI attracted a growing interest since the discovery of their role in insulin-activated signaling pathways and in the physiopathology of metabolic syndrome and type II diabetes. Insulin stimulates glucose uptake from bloodstream and glucose oxidative and nonoxidative disposal by activation of glycogen synthase (GS) and mitochondrial PDH. The dominant paradigm involves the activity of the insulin receptor tyrosine (Tyr) kinase and its primary phosphorylated substrate, the insulin receptor substrate (IRS) family [45]. However, a number of studies have shown that transduction signaling activated by insulin receptors also involves cytoplasmic second messengers generated in parallel with the phosphorylation events initiated by Tyr kinase [46].

Two separate IPG were purified from rat liver: one (containing DCI and galactosamine) was able to activate pyruvate dehydrogenase phosphatase and the second (containing MI and glucosamine) was able to inhibit cyclic adenosine monophosphate (cAMP) kinase and adenylate cyclase, both regulatory enzymes in FFA metabolism [47].

Pioneering studies identified inositol glycans generated from *Trypanosoma brucei*, which displayed insulin mimetic action inhibiting lipolysis, glucose 6 phosphatase, and fructose-1,6 diphosphatase, well-known insulin-mediated metabolic effects [48]. *In vivo* and *in vitro* studies later confirmed this hypothesis, showing that inositol glycans had insulin mimetic effects in human ovarian theca cells and stimulated testosterone biosynthesis [49, 50].

A recently proposed hypothesis is that insulin activates its specific Tyr kinase receptor which autophosphorylates itself and recruits IRS proteins, membrane adaptor proteins coupling insulin receptors to their intracellular signaling mechanisms. The first target of this pathway is phosphatidylinositol-3 kinase (PI3K), generating phosphatidylinositol 1,4,5 triphosphates (PIP3) which, in turn, activates the phosphoinositide-dependent kinase (PDK) kinase to phosphorylate and activate Akt kinase, leading to glucose transporter type 4 (GLUT4) translocation, GS activation, and stimulation of mammalian target of rapamycin (mTOR) kinase. At the same time, insulin receptor is coupled to the G-protein Gq that activates a phospholipase allowing the release of second messengers from GPI lipids (DCI glycan). These second messengers then activate phosphatase 2Cα (PP2Cα), which directly dephosphorylates and activates glycogen synthase and indirectly activates GS via PI3K. The same messengers inside mitochondria might activate pyruvate dehydrogenase phosphatase (PDHP), thereby activating pyruvate dehydrogenase and oxidative glucose metabolism [51].

Consistent with the proposed role of chiro-inositol as second messengers in insulin-mediated effects, other studies have shown that in muscle and urinary samples from type II diabetic patients DCI levels were about 50% lower than in samples from control subjects, whereas MI levels were not significantly different from control [52]. The difference observed in type II diabetes samples, with lower DCI and higher MI levels, was called inositol imbalance. The low DCI level and the inositol imbalance seemed to be related to the amount of insulin-resistance, worsening from normal to impaired glucose tolerance to type 2 diabetes [53]. Insulin-resistance was inversely related to urinary D-chiro-inositol excretion. Interestingly, women with PCOS had a blood deficiency of DCI and normal MI levels compared to control subjects [54]. The decreased production of chiro-inositol glycans observed during insulin-resistance might be explained by either deficiency of chiro-inositol or by decreased release mechanism [51]. On the other side, inositol imbalance might be partially explained with epimerase function failure: in fact, in a rat model of type II diabetes, conversion from MI to DCI in insulin-sensitive tissues was found to be reduced from 20 to 30% to 5% compared to normal rats [55]. Epimerase is also controlled by insulin that stimulates this enzyme to synthetize DCI starting from MI [56].

MI has been suggested to play a key role in oocyte fertilization: different studies describe a correlation between oocyte quality and follicular fluid concentrations of myo-inositol both in *in vitro* mouse models and on human oocytes studied during *in vitro* fertilization procedures [39, 57].

Intracellular calcium release mechanisms are accurately modulated during oocyte maturation and maximal sensitivity of calcium release is acquired during final stage of oocyte maturation. The PtdIns signaling pathway through inositol triphosphate (insP3) seems to regulate this oocyte maturation stage [58, 59]. Depletion of inositol may desensitize PtdIns by slowing down the resynthesis of precursors, as proposed by some authors [60].

The ovaries are sensitive to insulin and according to DCI ovary paradox theory, increased DCI level, due to increased epimerase function within the ovaries, is associated with a local MI deficiency and poor oocyte quality [61]. This failure had negative effects in FSH stimulation and in ovulation. Some authors showed that high (unnecessary?) dosage of DCI supplementation may damage oocytes [62]. In accordance with this finding, the conclusions of the "International Consensus Conference on Myo-inositol and

D-Chiro-inositol in Obstetrics and Gynecology" [63] also emphasized the negative effect of increasing doses of DCI on the ovary. However, the upper limit for DCI may prove to be higher in future studies if MI is supplemented simultaneously with DCI.

5. Clinical Data

The use of inositol administration to patients with PCOS found a rationale in the numerous physiological functions that these molecules regulate, as discussed above.

In literature, a complete consensus was not reached about inositols dosage to be used and which inositol isoforms are more active to improve symptoms and biochemical rates in polycystic ovary syndrome. Another issue concerns the need for different therapeutic approaches according to principal signs and symptoms that the patient complains from, particularly if she wants to improve ovulation or ameliorate metabolic alteration or also reduce dermatological symptoms related to hyperandrogenism. One of the first studies carried out in overweight and obese patients with PCOS showed that the administration of DCI 1200 mg once daily for six to eight weeks led to a reduction of testosterone level, improved metabolic parameters, decreased insulin response to orally administered glucose, ameliorated systolic and diastolic blood pressure, triglycerides level, and ovulatory function [38]. In addition, our group recently confirmed these results, showing improved metabolic parameters, androgen levels, and dermatological sign of hyperandrogenism [64].

We already showed that the administration of either MI (4 g/die) or DCI (1 g/die) together with folic acid to two different groups of PCOS patients showed in both groups an improved systolic blood pressure, a reduction in circulating androgens levels, a reduction in LH and LH/FSH ratio, an increased insulin sensitivity evaluated through decreased HOMA index, and increased SHBG. According to our data analysis, we observed that MI seemed to have the most marked action on metabolic profile, whereas DCI mostly affected hyperandrogenism parameters [65]. Furthermore, both groups showed ameliorated regularity in menstrual cycles without any significant difference between the two inositol isoforms.

Other authors confirmed that administration of MI (2 g daily) plus folic acid in overweight women with PCOS improved insulin-sensitivity, biochemical hyperandrogenism and regularity of menstrual cycles [66] similar to the effect obtained using insulin sensitizers such as metformin [67], congruently with previous studies which showed recovery of menstrual cycles and a reduction in insulin plasma level also after oral glucose tolerance test [68]. MI was also shown to improve oxidative stress in erythrocytes, in addition to improving metabolic and biochemical parameters in insulin-resistant women with normal weight and PCOS [15].

Other studies compared administration of MI (4 g daily) alone versus MI + DCI (3300 mg plus 84 mg) showing an improvement of metabolic markers in both groups, although the improvement was better in MI + DCI group [69, 70].

A recent review from our research group was proposed to evaluate the optimal dosage and which combination of MI and DCI should be used as a supplement in patients with PCOS according to a capillary analysis of literature [71]. Both MI alone (administered at a dosage from 2 g to 4 g daily) and DCI alone improved metabolic parameters related to insulin-resistance in patients with PCOS, regularized menstrual cycles, and reduced androgen levels and related symptoms. For a complete and comprehensive summary of the evidence reported in this chapter refer to Table 2.

6. Conclusion

Polycystic ovary syndrome is frequently associated with insulin-resistance and hyperinsulinemia. Insulin-resistance is correlated to genetic factors, environmental factors, and hormonal status. From literature, it is known that PCOS benefits from insulin-sensitizer therapy such as metformin.

Umpteen studies in the last thirty years have studied inositols and their role in cellular biology. These molecules take part in insulin transduction signaling pathway through generation of inositol phosphoglycans (IPG), particularly DCI-IPG, which act as second messengers with insulin-like functions. Inositols play a key role in many cellular functions as phosphatidylinositide production in response to hormonal-receptor binding (i.e., insulin and FSH) two important second messengers, DAG and insP3, which are involved in numerous cellular processes regarding differentiation and oocyte maturation. Besides, phosphoinositides are involved in many other functions such as the control of cellular structure and motility. In patients with PCOS, DCI is reduced, concurring to insulin-resistance and worsening the metabolic features of these patients. On the other side, reduced levels of MI impair oocyte quality, interfering with physiological follicular maturation.

Consistent with these findings, restoring inositols levels with oral supplementations ameliorates insulin-resistance, hyperandrogenism, regularity of menstrual cycles, and oocyte quality in patients with PCOS. Nevertheless, we strongly solicit future studies based on large cohorts, in order to clarify the pivotal role of inositol's isoforms in addressing the hormonal and metabolic parameters toward homeostasis in PCOS patients. In addition, we take the opportunity to propose a "tailored" dosage, based on the pretreatment conditions, which may allow us to improve the current knowledge about long-term outcomes in this kind of patients.

Disclosure

The authors alone are responsible for the content and writing of the paper.

Competing Interests

The authors have no proprietary, financial, professional, or other personal interest of any nature in any product, service, or company.

Table 2: Summary of the reported studies about myo-inositol and D-chiro-inositol use in PCOS treatment.

Study (first author, year)	MI and/or DCI dosage and duration	Main outcomes
Nestler, 1999 [38]	1200 mg of DCI/die versus placebo for six to eight weeks	In the group treater with DCI: (i) Area under the plasma insulin curve after the oral administration of glucose decreased (ii) Serum free testosterone concentration decreased (iii) Diastolic and systolic blood pressure decreased (iv) Plasma triglyceride concentrations decreased (v) Ovulatory rate increased
Laganà, 2015 [64]	1 gr of DCI/die plus 400 mcg of folic acid/die for 6 months	(i) Significant reduction of systolic blood pressure, Ferriman-Gallwey score, LH, LH/FSH ratio, total testosterone, free testosterone, Δ-4-androstenedione, prolactin, and HOMA index (ii) Significant increase of SHBG, glycaemia/IRI ratio, and menstrual cycle regularization
Pizzo, 2014 [65]	4 gr of myo-inositol/die plus 400 mcg of folic acid/die versus 1 gr of D-chiro-inositol/die plus 400 mcg of folic acid/die for six months	(i) MI compared to DCI decreased mostly systolic arterial pressure, LH/FSH ratio, total testosterone, D-4-androstenedione, prolactin, HOMA index, and, at the same time, SHBG considerably rises (ii) DCI compared to MI decreased more LH and free testosterone; at the same time, glycaemia/IRI ratio increased (iii) Both MI and DCI caused menstrual cycle regularization
Genazzani, 2008 [66]	MI 2 gr plus folic acid 200 mcg every day versus folic acid 200 mcg every day for 12 weeks	In the group treater with MI: (i) Plasma LH, prolactin, testosterone, insulin levels and LH/FSH resulted significantly reduced (ii) Insulin sensitivity expressed as glucose-to-insulin ratio and HOMA index resulted as significantly improved (iii) Menstrual cyclicity was restored in all amenorrheic and oligomenorrheic subjects
Gerli, 2003 [68]	100 mg, twice a day (=200 mg every day) of inositol (not specified if MI of DCI) versus placebo	(i) The ovulation frequency was significantly higher in the treated group compared with the placebo; the time in which the first ovulation occurred was significantly shorter (ii) The circulating concentration of E2 increased only in the inositol group during the first week of treatment (iii) Significant weight loss and leptin reduction were recorded in the inositol group (iv) Significant increase in circulating high density lipoprotein was observed only in the inositol treated group
Donà, 2012 [15]	MI 1200 mg/day versus placebo for 12 weeks	(i) MI treatment significantly improved metabolic and biochemical parameters (significant reductions were found in IR and serum values of androstenedione and testosterone) (ii) A significant association between band 3 tyrosine phosphorylation levels and insulin area under the curve was found at baseline but disappeared after MI treatment, while a correlation between band 3 tyrosine phosphorylation and testosterone levels was detected both before and after MI treatment
Nordio, 2012 [69]	550 mg MI + 13,8 mg DCI/day versus 2 gr MI/day for 3 months	(i) Plasma glucose and insulin concentrations showed a significant reduction in the MI + DCI group while no relevant changes were reported in the treatment with MI alone (ii) Compared to the MI group, the decrement of total testosterone and the increment of the serum SHBG were more relevant in MI + DCI group
Minozzi, 2013 [70]	550 mg MI + 13,8 mg DCI/day	(i) Improved LDL levels, HDL, triglycerides, and HOMA-IR.

MI: myo-inositol; DCI: D-chiro-inositol; LH: luteinizing hormone; FSH: follicle-stimulating hormone; SHBG: sex hormone binding globulin; E2: estradiol; HOMA: Homeostasis Model Assessment; IRI: glycaemia/immunoreactive insulin; IR: insulin-resistance; LDL: low density lipoprotein; HDL: high density lipoprotein.

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Clinical Study

A Combined Therapy with Myo-Inositol and D-Chiro-Inositol Improves Endocrine Parameters and Insulin Resistance in PCOS Young Overweight Women

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Introduction. We evaluated the effects of a therapy that combines myo-inositol (MI) and D-chiro-inositol (DCI) in young overweight women affected by polycystic ovary syndrome (PCOS), characterized by oligo- or anovulation and hyperandrogenism, correlated to insulin resistance. *Methods.* We enrolled 46 patients affected by PCOS and, randomly, we assigned them to two groups, A and B, treated, respectively, with the association of MI plus DCI, in a 40 : 1 ratio, or with placebo (folic acid) for six months. Thus, we analyzed pretreatment and posttreatment FSH, LH, 17-beta-Estradiol, Sex Hormone Binding Globulin, androstenedione, free testosterone, dehydroepiandrosterone sulphate, HOMA index, and fasting glucose and insulin. *Results.* We recorded a statistically significant reduction of LH, free testosterone, fasting insulin, and HOMA index only in the group treated with the combined therapy of MI plus DCI; in the same patients, we observed a statistically significant increase of 17-beta-Estradiol levels. *Conclusions.* The combined therapy of MI plus DCI is effective in improving endocrine and metabolic parameters in young obese PCOS affected women.

1. Introduction

Polycystic ovary syndrome (PCOS) is a heterogeneous syndrome, involving a growing number of women in reproductive age, diagnosed on the basis of three different factors: oligo- or anovulation, clinical/biochemical hyperandrogenism, and polycystic ovary, with the presence on ultrasound of ≥12 follicles in each ovary measuring 2 ± 9 mm in diameter and/or increased ovarian volume (>10 mL) [1, 2]. PCOS affected patients that had menstrual irregularity, followed in many cases by infertility [3, 4] and mood disorders, such as anxiety and depression [5]. Though PCOS pathogenesis still remains unclear, insulin resistance (IR) and the consequential hyperinsulinemia are considered primary triggers, both in obese and in lean women with this syndrome [6–8]. Indeed, hyperinsulinemia induced by IR occurs in roughly 80% of PCOS obese women, as in 30–40% of PCOS lean women [9], suggesting that IR is independent but also exacerbated by obesity; this latter considered an enhancing factor that positively correlates with the multifactorial syndrome [10–12]. It was hypothesized that in patients with PCOS altered insulin signaling may generate the IR which in turn causes abnormal ovarian steroidogenesis [13, 14], so that several insulin-sensitizing compounds have been proposed as possibly safe and efficacious long-term treatment of PCOS [15]. Among these drugs, metformin resulted to be the most used and studied drug, even if this molecule is predominantly associated with gastrointestinal discomforts consisting of bloating, nausea, and diarrhea [14, 16]. Interesting and promising results have been obtained focusing on two inositol stereoisomers, such as myo-inositol (MI) and D-chiro-inositol (DCI), acting like insulin mediators [17–19]. As insulin second messengers, both these molecules are involved in increasing insulin sensitivity of different tissues to improve metabolic and ovulatory functions [20]. In particular, at low dosage, DCI restores normal insulin sensitivity in the typical insulin target tissues, reducing the circulating insulin and androgens and inducing an enhancement in ovulation frequency. On the contrary, MI exerts its beneficial effects mainly at the ovary level, where it is highly concentrated,

both enhancing insulin pattern and also directly acting on a number of ovarian functions, including steroidogenesis [21]. Some authors [22] postulated that PCOS affected women, with IR, presenting in the ovary a misbalance in MI/DCI ratio, resulting in DCI overproduction and in turn in a deficiency in MI, which would explain the excessive androgen biosynthesis. Other authors [23], instead, proposed that the increased androgen levels in PCOS patients might be linked to a decreased MI/DCI. In a recent study, Facchinetti et al. [24] discovered that the physiological MI/DCI ratio was 40 : 1 and, based on this finding, as well as on the specific behavior of both stereoisomers, we investigated the effects of a therapy that combines MI plus DCI in the ratio of 40 : 1, versus placebo, in order to improve some clinical outcomes in PCOS young overweight women.

2. Methods

2.1. Patients and Study Design. This randomized controlled trial enrolled 46 obese women with BMI > 30 who were affected by PCOS according to Rotterdam criteria [1, 2]. All the women were enrolled at the Department of Clinical and Experimental Medicine, University of Pisa. Patients with diabetes, smokers, and alcohol users were ruled out from the study. After all patients subscribed their written informed consent to be involved into the study, they were randomly assigned to two groups, A and B. At baseline, patients in groups A and B did not differ significantly. In group A, 21 women received MI plus DCI combined treatment at the ratio of 40 : 1 (the physiologic ratio of the two isomers in the body) in soft gel capsule containing 550 mg of MI, 13.8 mg of DCI, and 200 μg of folic acid (INOFOLIC® COMBI, LO.LI.PHARMA) twice a day. Group B, with 25 women, received the same amount of folic acid (200 μg) as placebo twice a day. The treatments were performed for six months. At the beginning of the study, all the patients were in the follicular phase of the menstrual cycle.

2.2. Study Measurements. All patients were evaluated for FSH, LH, 17-beta-Estradiol (E), Sex Hormone Binding Globulin (SHBG), androstenedione, free testosterone, and dehydroepiandrosterone sulphate (DHEAS) levels at the baseline and after the six months of therapy with MI plus DCI association or with placebo. FSH and LH serum levels were detected by immune-enzymatic assay (Access Immunoassay System, hLH, hFSH, Beckman Coulter, Brea, CA, USA). Estradiol levels were measured by competitive immunoassay (Access Immunoassay System, Estradiol, Beckman Coulter, Brea, CA, USA). SHBG levels were detected by immunoassay (Access Immunoassay System, SHBG, Beckman Coulter, Brea, CA, USA). Serum levels of androstenedione were measured by conventional immune-enzymatic assay (Access Immunoassay System, androstenedione, Beckman Coulter, Brea, CA, USA). Free testosterone serum levels were measured by immune-enzymatic assay (Access Immunoassay System, free testosterone, Beckman Coulter, Brea, CA, USA). DHEAS was measured by conventional immunoassay (Access Immunoassay System, DHEAS, Beckman Coulter, Brea, CA, USA).

Table 1: Characteristics of patients who received MI plus DCI (group A) or placebo treatment (group B).

	Group A (n = 21)	Group B (n = 25)
Age (years)	23 ± 6.8	25 ± 7.3
Height (cm)	164 ± 6.7	168 ± 6.9
Weight (kg)	85 ± 13.5	88 ± 14
BMI	32 ± 4.8	31 ± 4.6

BMI: body mass index.

Insulin resistance was measured by means of Homeostasis Model Assessment (HOMA) in addition to determining fasting glucose and insulin with the same timeline and modalities. Blood samples, taken at the baseline and after the six-month treatment period under similar conditions, were separated by centrifugation at 2000 ×g for 15 minutes at 4°C, and the serum obtained was stored at −20°C within one hour of collection. Before the analysis, all the serum samples were thawed and entirely mixed.

2.3. Statistical Analysis. Data reported indicate mean values ± standard deviation (SD). Paired t-test was used to identify the differences between variables at baseline and after six months of treatment with MI plus DCI or with placebo, respectively. Differences were considered statistically significant at p value <0.05.

3. Results and Discussion

The goal of this study was to investigate if the therapy combining MI and DCI in the ratio of 40 : 1 could improve the endocrine profile and the insulin resistance of obese women with a PCOS diagnosis. To address this issue, 46 young obese patients affected by this syndrome, whose characteristics are summarized in Table 1, were randomly included in two groups and then treated with MI plus DCI at the ratio of 40 : 1 with or placebo for six months. Insulin resistance, evaluated as HOMA index, fasting insulin, and fasting glucose, and also hormonal parameters were determined at the baseline and after the six-month therapy. As shown in Table 2, we observed that, with respect to the baseline values, only the combined therapy of MI plus DCI significantly rebalanced the endocrine and metabolic profiles of these patients, ameliorating their insulin resistance and the ovulatory function, as successfully recorded by ultrasound. As a matter of fact, LH and free testosterone levels decreased after the combined treatment, downregulating the hyperandrogenism, and even HOMA index and fasting insulin, markers of insulin resistance, resulted to be significantly reduced. On the other hand, E and SHBG significantly increased, showing restoring in ovulation capability. No relevant changes in these sex hormones were reported in group B, treated with placebo, and no significant modifications were observed after the treatment in both groups A and B for what concerns BMI, FSH, androstenedione, DHEAS, and fasting glucose. Importantly, no relevant side effect was recorded during the combined therapy with MI plus DCI. Overall, these results

Table 2: Baseline and posttreatment endocrine and metabolic parameters of groups A and B of PCOS patients.

	Group A ($n = 21$)			Group B ($n = 25$)		
	Baseline	MI plus DCI	p value	Baseline	placebo	p value
FSH (mIU/mL)	5.86 ± 1.75	4.96 ± 1.74	ns	5.67 ± 1.11	5.47 ± 0.63	ns
LH (mIU/mL)	12.5 ± 8	8.5 ± 4.04	$p < 0.05$	11.27 ± 7.2	11.25 ± 5.35	ns
E (pg/mL)	47.06 ± 18.20	107.42 ± 92.86	$p < 0.01$	50.37 ± 19.45	52 ± 20.2	ns
Fasting insulin (μU/mL)	20.19 ± 8.14	10.74 ± 5.46	$p < 0.001$	18 ± 8	17.8 ± 8.2	ns
Fasting glucose (mg/dL)	85 ± 5.96	86 ± 7.12	ns	86.2 ± 9.1	84.73 ± 8.3	ns
Free testosterone (ng/dL)	0.76 ± 0.20	0.62 ± 0.15	$p < 0.05$	0.85 ± 0.22	0.83 ± 0.2	ns
SHBG (nmol/L)	24.11 ± 10.35	35.85 ± 24.3	$p < 0.05$	20.44 ± 8.77	21.36 ± 7.57	ns
Androstenedione (ng/mL)	4.25 ± 1.48	4.01 ± 1.70	ns	3.48 ± 1.21	3.12 ± 2.23	ns
DHEAS (μg/dL)	327.32 ± 150.89	347.6 ± 170.98	ns	337.95 ± 155.79	315.83 ± 145.59	ns
HOMA	3.38 ± 1.97	1.97 ± 1.48	$p < 0.05$	3.48 ± 2.02	2.8 ± 1.4	ns

E, 17-beta-Estradiol; P, progesterone; 17OHP, 17-OH-progesterone; SHBG, Sex Hormone Binding Globulin; DHEAS, dehydroepiandrosterone sulphate.

demonstrated the clinical importance of a combined therapy of MI plus DCI to correct the PCOS metabolic and reproductive aspects and they are largely in agreement with the issues discussed on the two international consensus conferences on MI, DCI, and their link with PCOS [25, 26]. PCOS is a syndrome whose pathogenesis remains still largely unclear, even though several etiological factors are demonstrated to be involved. Compelling evidences claimed the pivotal role of insulin resistance and/or compensatory hyperinsulinemia in this syndrome [9, 27–29]; indeed they tightly contribute both directly (increasing the ovarian production of androgens) and indirectly (modulating the hepatic SHBG synthesis) to hyperandrogenism development, one of the main features of those patients affected by PCOS [30, 31], especially in case of overweight women [32]. Nevertheless, literature findings consistently demonstrated that a deficiency in the tissue availability and/or usage of MI and/or DCI in women diagnosed with PCOS could likely concur to the IR typical of this syndrome [22, 23]. The two inositol stereoisomers, MI and DCI, acting as insulin-sensitizers, have been demonstrated to positively influence the clinical history of PCOS patients, ameliorating their endocrine and metabolic profile both alone and in combination [19, 33–37]. DCI alone, at low dosage, may restore normal insulin sensitivity in the typical insulin target tissues, inducing an enhancement in ovulation frequency which could be ascribed to the general improved insulin sensitivity and to the reduced circulating insulin and androgens. On the contrary, MI exerts its beneficial effects mainly at the ovary level, both enhancing insulin pattern and also directly acting on a number of ovarian functions, including steroidogenesis [22]. The ability of both inositol stereoisomers to regulate glucose metabolism in a different manner (DCI promotes glycogen synthesis, while MI may support glucose cell intake) [38] is mirrored by their different concentration in the tissues: while DCI is highly concentrated in glycogen storage tissues (liver, muscles, and fat), MI is more abundant in those tissues that need a large amount of glucose, such as brain, heart, or ovary [39]. From this knowledge, a combined therapy with MI plus DCI in their physiological plasma ratio (MI/DCI 40 : 1) seems to be the most appropriated clinical approach to integrate the positive effects exerted by both inositol stereoisomers.

4. Conclusions

The data reported are encouraging and they offer therapeutic options to the first-line treatments in PCOS women with moderate or severe hyperandrogenism and/or menstrual abnormalities, which are represented by metformin as well as by oral contraceptives. These compounds effectively suppress LH release and the consequent androgen production from the ovary; also they increase the sex hormone binding protein synthesis, lowering the levels of circulating free androgens [40]. Unfortunately, if the patient aims to restore ovulation in order to conceive, contraceptives are not the clinical strategy to follow. Furthermore, prolonged use of contraceptives may increase homocysteine levels after six months of treatment [41], as well as the risk of venous thromboembolism [42]. For what concerns metformin, several side gastrointestinal effects (diarrhoea, nausea, vomiting, and abdominal bloating) and metabolic complications have been evidenced after a long-term treatment [43]. For all these reasons, even though more studies on a higher number of patients and with greater statistical significance are needed to confirm these striking posttreatment outcomes, safe combined use of inositol stereoisomers should be largely suitable and it might represent a valid clinical approach in PCOS management.

Abbreviations

DCI: D-Chiro-inositol
DHEAS: Dehydroepiandrosterone sulphate
E: 17-Beta-Estradiol
HOMA: Homeostasis Model Assessment
IR: Insulin resistance
MI: Myo-inositol
PCOS: Polycystic ovary syndrome
SHBG: Sex Hormone Binding Globulin.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Clinical Study

Treating Woman with Myo-Inositol Vaginal Suppositories Improves Partner's Sperm Motility and Fertility

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Motility is the feature that allows spermatozoa to actively reach and penetrate the female gamete during fertilization. When this function is altered, and especially decreased, troubles in conceiving may occur. In this study, we demonstrated that treating fertile women with myo-inositol (MI) vaginal suppositories ameliorated their partners' sperm motility and also positively affected their conceiving capacity, without changes in cervical mucus structural and biochemical characteristics. Indeed, by means of the postcoital test on female cervical mucus, a significant improvement especially in progressive sperm motility was recorded after MI suppository use. Concomitantly, after MI treatment, a reduction of immotile spermatozoa percentage was observed. Importantly, MI vaginal supplementation positively correlated with a pregnancy for 5 of the 50 couples enrolled in the study, leading us to speculate that this substance may substantially contribute to create in the cervical mucus an ideal *milieu* that makes spermatozoa more motile and functionally able to fertilize. Even though the detailed mechanism is still unclear, these results should encourage MI vaginal use for the clinical improvement of male infertility, through their partners.

1. Introduction

Infertility is a medical, psychological, and social problem of couples in reproductive age [1]. Male factor issues play an etiologic role in a substantial portion (10–15%) of couples with conceiving troubles [2].

Different causes may conduct to male infertility: varicocele, cryptorchidism, and hypogonadism are the most frequently observed; on the other hand, in several cases infertility is determined by sperm parameters below the World Health Organization (WHO) reference values [3].

In particular, sperm motility is a critical factor in determining semen quality and fertilizing ability, considered as potential for movement, cervical mucus penetration, capacitation, zona recognition, acrosome reaction, and sperm-oocyte fusion [4–7].

In vitro studies evidenced that inositols, and mainly the prevalent isomer myo-inositol (MI), are sugars strongly involved both in spermatozoa maturation and in their migration from the epididymis [8–11], suggesting a potential role for these molecules in affecting sperm motility and fertility.

A recent prospective double-blind randomized placebo controlled study determined that MI improves sperm parameters and serum reproductive hormones in patients with idiopathic infertility [12].

On these findings, we determined if male mild infertility, assessed by spermiogram as reduced sperm motility in respect to WHO criteria, could be improved after treating the fertile female partner with MI vaginal suppositories (Xyminal®; Lo.Li Pharma s.r.l., Rome, Italy).

By the postcoital test on female cervical mucus, we investigated if male sperm motility parameters, especially progressive motility and immotility, could be ameliorated after female MI vaginal use. Indeed, MI may contribute to create a more comfortable environment to facilitate sperm progression and sperm-oocyte cross-talk. The number of couples who ameliorated their fertility after treatment, acquiring conceiving ability, was also registered.

2. Materials and Methods

2.1. Study Population and Inclusion/Exclusion Criteria. For this study 50 couples who did not achieve a pregnancy after at least 1 year of unprotected intercourse with the same fertile partner were enrolled.

In particular, male patients included showed a mild infertility with troubles in conceiving from 12 to 36 months. Their infertility was assessed by spermiogram according the WHO references and they all fell in the range between the 5th and the 40th percentile, with motility lower than WHO values (40% sperm with progressive, slowly progressive and nonprogressive movement, and 32% sperm with progressive and slow progressive movement) [3].

All female partners were fertile and able to be pregnant.

Men with azoospermia, severe oligozoospermia, or an identifiable cause of infertility were excluded from the study, as well as couples where sterility was due to the female partner.

All the couples were under treatment at the IVF Unit (Villa Mafalda Clinic, Rome, Italy) and they all have signed an Informed Consent Form.

2.2. Protocol Design and Treatment. The primary outcome of this study was to investigate if MI vaginal suppositories, given to fertile females, can ameliorate the impaired motility and poor quality of their partners' sperm.

The secondary outcome was to test if MI vaginal suppositories can increase the number of pregnancies in these couples.

This case-control open label study used two-phase strategies, lasting two menstrual cycles. In the first stage, at the 9th day of female menstrual cycle, a baseline spermiogram analyzing sperm parameters was performed on male partners, according to WHO references [3]. Sperm motility (% of total motility, progressive motility, not progressive motility, and static spermatozoa) was assessed. Concomitantly, between the 9th and the 11th day of the menstrual cycle, ultrasound monitoring of female ovulation was followed, and the day after leading follicle was >18 mm couple was requested to have intercourse. The postcoital test on female partner was performed 3–6 hours after the intercourse.

In the second stage, in correspondence with the 9th day of female menstrual cycle, a second spermiogram was performed on male partners and the ultrasound monitoring of female ovulation was followed. When the leading follicle was >16 mm, female partners started the treatment with MI vaginal suppositories (2 mg of MI, Xyminal; Lo.Li Pharma s.r.l., Rome, Italy). The treatment consisted of one vaginal suppository for three consecutive days in the periovulation period (i.e., from the 11th to the 13th one of a regular menstrual cycle). The morning subsequent to the administration of the last suppository, after verifying leading follicle was >18 mm, couples were requested to have the intercourse and 3–6 hours later the postcoital test was performed.

2.3. Sperm Analysis. Semen samples were obtained after 3 days of abstinence. They were collected into sterile containers and allowed to liquefy at 37°C for 30 min. A routine sperm analysis was carried out according to WHO criteria [3].

Table 1: Patients' baseline average features.

	Age	Weight (Kg)	Height (cm)	BMI
Females ($n = 50$)	34.5 ± 3.6	57.6 ± 4.2	167.3 ± 5.7	23.1 ± 1.8
Males ($n = 50$)	37.1 ± 4.1	78.4 ± 3.9	178.6 ± 6.9	25.2 ± 1.6

Mean ± SD
BMI, body mass index.

Nonprogressive motility (all other patterns of motility with an absence of progression), progressive motility (spermatozoa moving actively, either linearly or in a large circle, regardless of speed), total motility (nonprogressive motility + progressive motility), and static spermatozoa were determined.

2.4. Postcoital Test (PCT). PCT was performed 3–6 hours after coitus, as described by Hull et al. [13] and according to the WHO procedure [3]. Briefly, we exposed the cervix with a speculum and gently wiped the external os with a cotton swab to remove the external pool of vaginal contaminants. We removed the exocervical mucus with the swab or with forceps and collected cervical mucus from the endocervical canal by aspiration with a tuberculin syringe (without needle).

We advanced the tip of the device approximately 1 cm into the cervical canal before applying suction. Then, we maintained suction as the device was withdrawn. Just before the device was completely withdrawn from the external cervical os, we released the suction pressure.

The evaluation of the cervical mucus properties included the assessment of spinnbarkeit, ferning (crystallization), viscosity, and pH, according to the system devised by Moghissi [14].

Sperm total and progressive and nonprogressive motility were evaluated following WHO criteria [3].

2.5. Statistical Analysis. The results are presented as mean ± standard deviation (SD). Differences were statistically analyzed with Student's paired t-test. $p < 0.05$ was considered statistically significant.

3. Results

For this study 50 couples were enrolled; for each couple, the man was affected by mild infertility while the woman was fertile and able to conceive. Patients' average baseline characteristics are summarized in Table 1.

Spermiogram showed that sperm motility, as well as the other sperm parameters, did not change between the first and second phase of the study, as reported in Table 2.

On the other hand, the postcoital test performed on cervical mucus showed an improvement in sperm total motility, after MI supplementation (Table 3). In particular, sperm progressive motility resulting significantly increased ($p < 0.001$), compared to nonprogressive motility, and the number of immotile spermatozoa decreased as well.

Table 2: Sperm parameters by spermiogram.

	1st stage	SD	2nd stage	SD	p value
Total motility (PR + NP) (%)	30.2	±11.2	30.3	±10.8	NS
PR (%)	15.3	±8.5	15.9	±8.0	NS
NP (%)	14.9	±5.3	14.4	±5.4	NS
IM (%)	69.8	±15.8	69.7	±16.8	NS
Semen volume (mL)	2.72	±0.61	2.5	±0.63	NS
Total sperm number (10^6 per ejaculate)	72.0	±28.37	70.2	±25.23	NS
Sperm concentration (10^6 per mL)	25.0	±9.5	23.0	±8.7	NS
Sperm morphology (normal forms, %)	21.0	±20.19	22.0	±20.4	NS

PR = progressive motility; NP = nonprogressive motility; IM = immotility.

Table 3: Postcoital test results on sperm parameters.

	Baseline	SD	+Xyminal	SD	p value
Total motility (PR + NP) (%)	40.8	±20.4	50.8	±11.1	$p < 0.05$
PR (%)	15.8	±10.6	29.0	±7.5	$p < 0.001$
NP (%)	25.0	±21.2	21.8	±6.7	NS
IM (%)	59.2	±20.4	49.2	±10.5	$p < 0.05$
Sperm number per HPF	3.0	±1.43	3.0	±1.53	NS

PR = progressive motility; NP = nonprogressive motility; IM = immotility; HPF = high-power field.

Table 4: Postcoital test results on cervical mucus.

	Baseline	SD	+Xyminal	SD	p value
Viscosity	2.7	±0.48	2.9	±0.37	NS
Spinnbarkeit	8.7	±1.05	8.6	±1.01	NS
Ferning	2.6	±0.49	2.6	±0.49	NS
pH	7.55	±0.3	7.3	±0.2	NS

PCT also evidenced that MI supplementation did not influence neither positively nor negatively the examined cervical mucus characteristics; indeed no significant differences were recorded after MI vaginal insertion in respect to the optimal baseline values, as reported in Table 4.

Moreover, as second clinical result, five couples were able to conceive after MI treatment.

Patients were kept under medical check for 30 days after the end of the treatment and no side effect was observed during or after the treatment with MI.

4. Discussion

Sperm dysfunction is one of the most commonly observed causes of infertility [15, 16]. Its primary manifestation is poor motility, which negatively impacts on conceiving [17–19]. Italian researchers firstly demonstrated that inositols and, in particular, MI *in vitro* improved sperm parameters, such as motility. Indeed, after incubation with MI, sperm progressive motility increased and the concentration of motile spermatozoa doubled as well, in both normozoospermic men and patients with abnormal sperm parameters [9–11]. Moreover, they observed an amelioration of sperm mitochondrial function that positively correlates with total and progressive motility as well as with sperm quality [11, 20, 21].

On these bases, great attention to the ionic mechanisms and the involvement of protein kinases and phosphatases in regulation of sperm motility was paid. Ca^{2+} was found to be a key regulator, and elevated intracellular Ca^{2+} concentrations were required for both the initiation and maintenance of sperm motility [22–25]. Inositols binding opens calcium channels, increasing ionic intracellular concentrations in the flagellum [26]. Also MI, through PKA-, PKB-, and PKC-dependent pathways, modulates intracellular Ca^{2+} concentrations by acting on the sperm plasma membrane, mitochondria, acrosome, and neck region [27–31]. Thus, Ca^{2+} release is mandatory for all the events that allow the spermatozoa to digest the zona pellucida and penetrate into the oocyte in order to fertilize it [6].

These findings were confirmed by a recent *in vivo* study, in which Calogero and colleagues showed that MI improved sperm parameters, such as motility, and serum reproductive hormones in patients with idiopathic infertility, confirming that MI supplementation should be encouraged in these patients [12].

Following these important clinical results, our aim was to investigate if men with mild infertility could benefit from the treatment of their fertile female partner with MI vaginal suppositories.

Indeed, on a total of 50 couples we intended preliminarily to understand if a supplemented vaginal environment could ameliorate sperm quality and positively modulate sperm motility parameters and their conceiving ability.

To address this issue, two postcoital tests (PCT) were performed in the two stages of the trial and results were compared showing interesting difference after MI vaginal use in terms of spermatozoa motility, evaluated as total, progressive, and nonprogressive motility and as immotility percentage.

The aims of a PCT are to determine the number of active spermatozoa in the cervical mucus and to evaluate sperm

survival [32] and sperm behavior some hours after coitus (the reservoir role of mucus) [14].

Anyway, the use of PCT in the basic fertility workup has been subject to debate for so long [33–37]. The conclusion was that this test is an effective predictor of conception, if defined female causes of infertility are absent and duration of infertility is less than 3 years [38].

In particular, our PCT recorded that progressive motility greatly increased and, concomitantly, the number of immotile spermatozoa decreased after MI vaginal supplementation to women. These data did not depend on changes in their cervical mucus structural and biochemical features after MI treatment. In fact, these parameters resulted as being quite unmodified in respect to baseline values. Therefore, we hypothesized that the interaction between spermatozoa and cervical mucus microenvironment could be responsible for the amelioration in sperm motility, determining the ideal conditions to trigger those mechanisms ending with protein kinases activation and intracellular Ca^{2+} release, mentioned above.

As second important result, we evidenced that the treatment has led to a pregnancy for five couples. This interesting result may support the positive correlation MI supplementation-sperm motility-fertility and should be further verified in a larger cohort study.

Despite the intrinsic limitations of the study (i.e., low number of patients which slightly reduced the study's statistical power), overall the data herein reported are encouraging and promote the successful clinical use of MI in women to positively affect their partners' sperm motility and infertility.

Further studies are needed to better clarify *in vivo* the cross-talk between spermatozoa and cervical mucus, in order to understand the mechanisms that MI might trigger and control to improve fertility outcome.

Competing Interests

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

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