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The myo-inositol effect on the oocyte quality and fertilization rate among women with polycystic ovary syndrome undergoing assisted reproductive technology cycles: a randomized clinical trial

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Abstract

Purpose The aim of the present study was to evaluate the effect of myo-Inositol administration on oocyte quality, fertilization rate and embryo quality in patients with PCOS during assisted reproductive technology (ART) cycles.

Methods Fifty infertile PCOS patients were randomly designated in two groups. In the study group, patients received daily doses of 4 g myo-Inositol combined with 400 mg folic acid and in the control group patients received only 400 mg folic acid from 1 month before starting the antagonist cycle until the day of ovum pick up. Oocyte and embryo qualities were assessed according to European Society of Human Reproduction and Embryology (ESHRE) guidelines. The gene expression of PGK1, RGS2 and CDC42 as a factor of oocyte quality in granulosa cells was analyzed using real-time RT-PCR. Levels of total antioxidant capacity (TAC) and reactive oxygen species (ROS) were evaluated by chemiluminescence assay in follicular fluid.

Results The percentage of metaphase II oocyte, fertilization rate and embryo quality significantly improved in the study group ($p < 0.05$), but the number of retrieved oocytes and follicle count were not statistically different between groups. Furthermore, the gene expression of PGK1, RGS2 and CDC42 was significantly higher in the study group ($p < 0.05$) but no differences were found between two groups in terms of TAC and ROS levels.

Conclusions The present study findings suggest that myo-Inositol alters the gene expression in granulosa cells and improves oocyte and embryo quality among PCOS patients undergoing ART.

Keywords Polycystic ovary syndrome · myo-Inositol · Oocyte · Fertilization · Assisted reproductive technology

Introduction

Polycystic ovarian syndrome (PCOS) with a prevalence of 6–15% is the most common endocrinopathy among women of reproductive age and accounts for approximately 75% of anovulatory infertility cases [1, 2]. Despite the higher number of oocytes retrieved from PCOS patients during an in vitro fertilization (IVF) treatment cycle, the quality and maturity of oocytes and embryo quality are lower than non-PCOS infertile women [3]. PCOS is defined by the presence of two of the following conditions: androgen excess, ovulatory dysfunction, or polycystic ovarian morphology. Ultrasound is required where either androgen excess or ovulatory dysfunction is not present. Some disorders including thyroid disease (thyroid-stimulating hormone), hyperprolactinemia (prolactin level), and non-classic congenital adrenal hyperplasia (serum 17-OHP) should be ruled out in all women with further assessment guided by clinical judgement [4]. In

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addition insulin resistance and hyperinsulinemia are common among these patients [5, 6].

Among different medical treatments, growing evidence suggests that insulin sensitizers decrease hyperandrogenism, and hyperinsulinemia in PCOS patients. myo-Inositol is an insulin-sensitizing supplement with antioxidant effects [7–9]. It has been suggested that the possible benefits of myo-Inositol administration for treatment of PCOS women may be due to its effect on intracellular metabolic processes, glucose metabolism [10], insulin sensitivity and oxidative stress [9, 11]. In recent years, limited studies have suggested that myo-Inositol can improve oocyte quality and fertilization outcomes in patients with PCOS [12, 13].

However, to the best of our knowledge, the effect of myo-Inositol on expression of genes related to oocyte quality has not been studied. Different genes including phosphoglycerate kinase 1 (PGK1), regulator of G-protein signaling 2 (RGS2), cell division cycle 42 (CDC42); which are related to developmental competence of the oocytes have been identified [14]. This study was conducted to evaluate the effect of myo-Inositol administration as an adjuvant on expression of some of the genes related to oocyte quality and oxidative stress parameters in the follicular fluid as well as oocyte maturation, fertilization rate and embryo quality in PCOS patients during ART cycle.

Materials and methods

This placebo-controlled double-blind randomized clinical trial was conducted in Shahid Akbar Abadi hospital IVF center, Iran University of Medical Sciences, Tehran, Iran, Feb 2017–May 2018. All infertile women referred to our center with PCOS based on Rotterdam criteria [15] who were candidate for IVF cycle, aged 20–35 years and with partner's normal semen analysis results (total volume > 1.5 cc, concentration > 15 million/ml and total motility > 40% as well as normal morphology > 4%, according to WHO 2010) were included in the present study. Patients were excluded if they had other metabolic diseases such as diabetes, BMI above 35, had allergy to myo-Inositol or had received any hormonal medications for at least 3 months before the start of the study. The sample size was calculated based on previous studies and the formula that estimates a sample size for comparison of two means with 80% power and 5% error type I, which necessitated at least 25 cases in each group [16, 17]. Taking into account the probable dropout (due to cycle cancelation, patient desire to exit the trial, etc.), the required minimum number of enrolled patients was determined to be 30 in each group (the rate of predicted dropout was 20%).

Patients were randomly designated in two groups using permuted block randomization. The size of blocks was four, six and eight. In the study group, patients received a daily dose of 4 g myo-Inositol combined with 400 mg of folic acid (Inofolic, Lo. Li. Pharma, Rome, Italy). Inofolic was given to the patients in the form of powder inserted in small pockets to be diluted into one standard glass of water and taken orally, from 1 month prior to IVF cycle until the day of ovum pick up. In the control group, patients only received a daily dose of 400 mg of folic acid from 1 month before starting the antagonist cycle until the day of ovum pick up. Folic acid powder was given to the subjects in the same manner as the case group. The sequence was developed by an independent biostatistician and implemented by the epidemiologist. All clinical investigators were blinded to the outcome determinations.

All participants signed written informed consent before being enrolled in the study and the study protocol was approved by the ethics committee of Iran University of Medical Sciences, Tehran, Iran (IRCT20171208037790N1).

Stimulation protocol

For ovarian stimulation, patients were administered oestradiol valerate at a daily dose of 4 mg for a period of 10 days before the onset of menstruation. Gonadotropins (Gonal-F®; Merck, Geneva, Switzerland) were administrated by starting dose of 150 IU from day 3 of the following cycle then GnRH antagonist (Cetrotide Merk Serono, Germany) was administered with a daily dose of 0.25 mg beginning when the largest follicles had reached a diameter of 13–14 mm. Ovulation was triggered using 0.2 mg GnRH agonist decapeptide (Ferring, Copenhagen NV) in the presence of at least two follicles 18–20 mm in size and oocytes were retrieved after 36 h under vaginal ultrasound guidance. We did not face any case of ovarian hyper-stimulation syndrome. The culture and insemination were performed in human tubal fluid medium (Cook IVF Medium, Brisbane, Australia) at atmosphere of 5% CO_2 and 37 °C. The IVF was performed using established techniques and only with mature oocytes. All embryos were frozen to transfer then in subsequent FET cycles.

Assessment of fertilization rate, pregnancy rate and embryo quality

Fertilization was assessed 16–18 h after IVF by observing two pronucleus and two polar bodies. Oocytes without visible pronucleus were considered to be unfertilized. Oocytes with a single pronucleus or more than two pronucleus were considered to be abnormally fertilized, and, therefore, were discarded.

Embryo quality was assessed approximately 44–48 h after IVF and was scored according to ESHRE guidelines. The grading criteria were as follows: grade 1, regular

blastomeres, fragmentation < 10% and no multi-nucleation; grade 2, regular blastomeres and fragmentation < 25% and multi-nucleation is not clear; grade 3, irregular blastomeres, fragmentation > 25% and presence of multi-nucleation [18].

Reactive oxygen species (ROS) and total antioxidant capacity (TAC) measurement

Follicular fluid was collected during oocyte retrieval and centrifuged for 7 min at 600 g. Clear supernatant was aliquoted and prepared for ROS measurements. ROS level was assessed by chemiluminescence assay, and luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione) was used as a probe. Total antioxidant capacity (TAC) in follicular fluid was measured by a commercial assay kit (TAC kit ZellBio GmbH, Ulm, Germany).

Isolation of mural granulosa cells (MGCs) from follicular fluid

MGCs were isolated from follicular fluid using 50% Percoll (Dorset—UK) gradient. After retrieval of oocytes, aspirated follicular fluid was centrifuged at 1000 g for 3 min at 21 °C. The pellet was solved in 4ml of phosphate buffer saline (PBS). The solution was added on 50% Percoll gradient and centrifuged at 400 g for 30 min at 21 °C. Using a Pasteur pipette, the layer between Percoll and FF was removed and washed with PBS again. Samples were then taken for extraction of RNA.

Real-time RT-PCR

Total RNA was extracted from mural granulosa cell samples by homogenization in 1 ml of TRIzol Reagent (Sigma-Aldrich), according to the manufacturer's instructions. All samples were treated with DNaseI (Fermentas, St. Leon Rot, Germany) to remove genomic DNA contamination. Samples were then analyzed spectrophotometrically using A260/A280 ratio method to determine RNA concentration, yield and purity. Total RNA was reverse transcribed using oligo dT primers (Metabion, Martinsried, Germany) and the Super Script First-Strand Synthesis System (200 U/ ml, Invitrogen). One reverse transcription control, which was performed using the conditions described above without SuperScript II enzyme was used in each PCR cycle. QPCR was used to quantify the mRNA transcript levels of PGK1, RGS2 and CDC42, with GAPDH mRNA transcripts as endogenous references. All experiments included negative controls with no cDNA. QPCR reactions were carried out as described previously [19]. Melting curves of PCR reactions were monitored to ensure the absence of contaminants or primer dimers. Standard curves were obtained for each gene using the logarithmic dilution series of cDNA. The threshold cycle values were normalized against the threshold value of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The qPCR data were analyzed using the comparative CT method.

Statistical analysis

All analyzes were performed using R software version 3.4.1. The distribution of quantitative variables was evaluated using Shapiro–Wilks test. The means of two independent variables were compared using *t* test or Mann–Whitney *U* tests based on the type of distribution. Analysis of variance (ANOVA) and Kruskal–Wallis test were used to compare the means of three or more groups. *P* values less than 0.05 were regarded as statistically significant.

Results

In this study, 60 infertile PCOS patients who were candidate for IVF cycles were randomly divided in two groups. Demographic and clinical characteristics of patients and sperm parameters of their husbands were comparable in the study group and the control group. We encountered no adverse side effects of myo-inositol in study group. At the end of study, 50 data from patients were analyzed since we had 5 drop-outs in each group of patients. The data are summarized in Table 1. In this table, non-significant difference in baseline characteristics confirmed the randomization effectiveness of the study. Total dose of gonadotropin and duration of ovulation induction did not decrease significantly in the study group (p value = 0.716 and p value = 0.92, respectively). Although the number of retrieved oocytes was not statistically different between two groups (p value < 0.6), the percentage of MII oocytes and fertilization rate (Table 2) were significantly increased in the study group compared with controls (p value = 0.0035 and p value = 0.03, respectively). In addition, the embryo quality was improved after treatment with myo-Inositol. The results showed that the percentage of grade 1 embryos was significantly increased and the percentage of bad embryos (grade 3) was significantly decreased in study group compared with the control group (Fig. 1a) (p value = 0.006 and p value = 0.029, respectively).

The gene expression of PGK1, RGS2 and CDC42 was measured as a factor of oocyte quality in granulosa cells. As shown in Fig. 1b, the gene expression level of these genes was significantly higher in the study group (p value = 0.013, p value = 0.021 and p value < 0.001, respectively) but no differences were found between two groups in terms of TAC and ROS levels (Fig. 2) (p value = 0.336 and p value = 0.433, respectively). The cumulative pregnancy rate was 40% among women who received myo-Inositol and 35% in the

Table 1 Anthropometric, endocrine, duration of infertility and sperm characteristics of the patients in treatment groups

Parameters	myo-inositol + folic acid (mean ± SD, $n = 25$)	Control (mean ± SD, $n = 25$)	p value
Age (years)	31.3 ± 4.1	29.78 ± 4.5	0.28
BMI (kg/m^2)	25.26 ± 5.2	26.2 ± 4.52	0.58
Duration of infertility (years)	6.45 ± 4.23	5.81 ± 4.47	0.65
FSH (mU/mL)	5.67 ± 2.37	6.07 ± 2.39	0.56
Prolactin (ng/mL)	33.35 ± 7	81.14 ± 24.27	0.66
TSH (mU/mL)	2.12 ± 0.88	2.07 ± 0.63	0.98
Total dose of Gonadotropin (IU)	1567.5 ± 422.09	1622.368 ± 510.03	0.716
Duration of stimulation (days)	12.6 ± 2.28	12.52 ± 2.48	0.92
Number of retrieved oocyte	13.8 ± 7.67	12.4211 ± 8.97	0.6
Estrogen level on triggering day (pg/ml)	5221.89 ± 8249.62	3735.84 ± 3312.98	0.49
Progesterone level on triggering day(ng/ml)	1.22 ± 0.83	0.8 ± 0.14	0.54
Sperm count (million)	50.68 ± 29.81	56.5 ± 36.38	0.59
Total sperm motility (%)	76.94 ± 21.918	40.27 ± 26.19	0.28
Normal sperm morphology (%)	7.68 ± 1.49	7.94 ± 1.66	0.61

The results are reported as mean + SD

BMI body mass index, *FSH* follicle-stimulating hormone, *TSH* thyroid-stimulating hormone

Table 2 The percentage of MII oocyte and fertilization rate in the study groups

	Inofolic (95% CI)	Control (95% CI)	p value
Rate of MII	78.67 (76.01, 81.33)	58.28 (53.97, 62.58)	0.003
Rate of fertilization	65.17 (62.59, 67.74)	46.85 (43.87, 49.83)	0.03

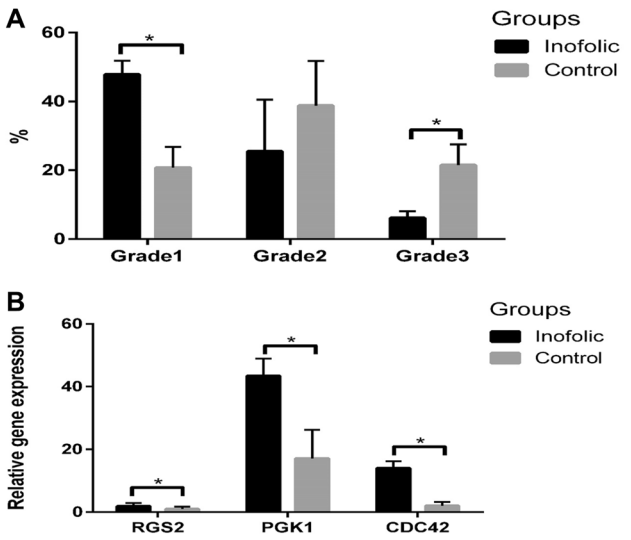

Fig. 1 **a** The percentage of grade 1 embryos was significantly increased (p value = 0.006) and the percentage of grade 3 embryos was significantly decreased (p value = 0.029) in study group compared with the control group, but the percentage of grade 2 embryos was not significantly different between two groups (p value = 0.059). **b** The gene expression level of RGS2 (p value = 0.021), PGK1 (p value = 0.013) and CDC42 (p value < 0.001) was significantly higher in the study group

control group, which did not indicate a significant improvement ($p = 0.744$).

Discussion

The results of the present study showed that myo-Inositol supplementation in ART cycles among patients with PCOS increases the percentage of MII oocytes to total oocytes, as well as the fertilization rate and the ratio of good quality embryos. In PCOS patients treated in ART cycles, despite the high number of oocytes, the quality of these oocytes and subsequent embryos was significantly lower [20], so a significant proportion of women suffering from PCOS need repeated ART attempts [21]. Previous studies demonstrated that total dose of gonadotropins and duration of stimulation days were reduced following treatment with myo-Inositol [22, 23], however, these results were not be confirmed by the present study. Although some studies have shown the positive effects of myo-Inositol supplementation among PCOS patients on the quality of oocytes and embryos [22, 24] but there are controversial opinions in this regard [25].

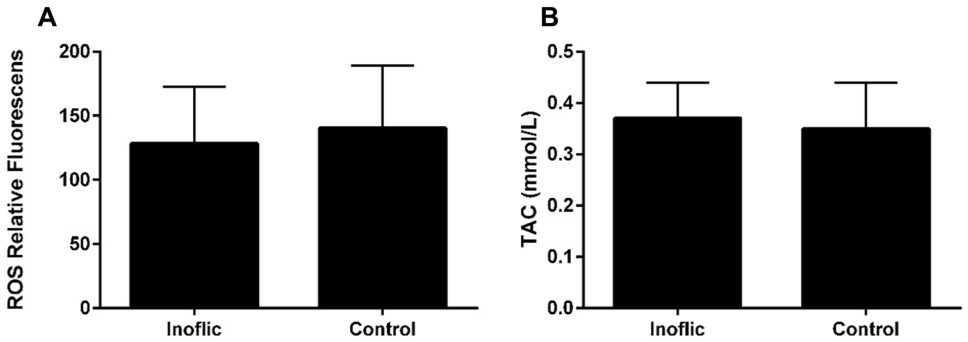

Fig. 2 **a** The levels of ROS (p value = 0.433) and **b** TAC (p value = 0.336) were not statistically different between two groups

In the present study, although the number of MII oocytes did not increase after the use of myo-Inositol, the ratio of MII oocytes to total oocytes was improved. Similar to our results, Vartanyan et al. showed that after myo-Inositol treatment in PCOS patients, the number of MII oocytes was even higher than those of normal women undergoing IVF due to male or tubal factors. Although in their study, there was no significant difference in the number of good embryos among PCOS patients who received myo-Inositol and normal women but the quality of the embryos improved following myo-Inositol treatment [13].

It is noteworthy that in our study, the number of grade 1 embryos among PCOS patients receiving myo-Inositol was significantly increased. This results were similar to those reported by Ciotta et al. [12]. However, the results of some other studies are not in line with these results.These differences could be due to the duration of the intervention or the type of ovarian stimulation protocols [20].

Despite several studies, the mechanism of myo-Inositol effect on the quality of embryo and oocyte has not yet been fully elucidated. Some studies suggest that myo-Inositol positive effects on the quality of embryo and oocyte may be due to its effect on enhancing insulin function [20, 26, 27]. myo-Inositol considered as an insulin sensitizers and its role in improving the metabolic status of patients with PCOS might be the reason for improving the quality of oocyte [28–30]. Zeng et al. in a meta-analysis study, showed that following myo-Inositol treatment among patients with PCOS, insulin resistance and estradiol levels are significantly reduced [31].

Hyperinsulinemia accelerate conversion of myo-Inositol to D-chiro-inositol (DCI), so it can lead to myo-Inositol deficiency and DCI overproduction. Recent studies demonstrated that increased dosage of DCI has a deleterious effect on oocyte quality [30, 32]. In vitro studies have also indicated that myo-Inositol may play an important role in oocyte maturity by increasing intracellular Ca^{2+} [26, 33]. In addition, the inositol antioxidant effects, along with methionine and alpha-lipoprotein, have been reported on the quality of embryos and oocytes [34, 35]. Oxidative stress plays an important role in follicle atresia and causes apoptosis in granulosa and oocyte cells [36].

In the present study, for the first time, the antioxidant effect of myo-Inositol alone was investigated and it was found that using myo-Inositol alone has no significant effect on reducing the amount of oxidants and increasing TAC in follicular fluid. As a result, myo-Inositol positive effects on oocyte quality and the resulting embryos in the present study were not due to the antioxidant role of myo-Inositol. Also, for the first time, we found that myo-Inositol has a significant effect on the expression of genes associated with oocyte quality in granulosa cells. Increasing the expression of RGS2, PGK1 and CDC42 genes following myo-Inositol treatment might indicate its effect on maturation and fertilization capacity of the oocyte [37]. Following ovarian stimulants, premature Ca^{2+} release prior to fertilization in MII oocyte is possible, which causes parthenogenesis and prevents fertilization in oocytes. One of the most important proteins that prevent Ca^{2+} increase is RGS2 [37, 38]. Therefore, increasing the fertilization rate following myo-Inositol treatment can be due to increased expression of this gene. Another gene affecting the quality of oocyte was PGK1, which is a transferase enzyme and plays an important role in glycolytic pathway. Glycolysis is critical for oocyte maturation and competence but in the final stages of folliculogenesis, oocytes are not able to oxidize glucose in the glycolysis process and are highly dependent on products of glycolysis provided by granulosa cells for energy supplementation [14, 39]. Therefore, increasing the expression of this gene in granulosa cells can improve glycogenesis in these cells, and this pathway can affect oocyte maturation and competence.

Finally the CDC42 gene is involved in a wide range of cell functions such as transcription, organization of cellular skeletons, apoptosis and cell proliferation [38]. In vivo studies have indicated that CDC42 has multiple roles in mouse oocyte maturation. Elevated apoptosis is observed in granulosa cells of PCOS patients and apoptosis of granulosa cells seems to have a negative effect on IVF outcomes [40]. A higher incidence of apoptotic granulosa cells has been related to poor embryo development, and low fertilization and pregnancy rates [41]. PCOS patients undergoing IVF procedure produce a high number of

oocytes but most of them have poor quality leading to poor outcomes [42]. Higher expression of CDC42 can decrease the apoptosis in granulosa cells and increase cell division, thus may improve the quality of follicles and oocytes [43]. Over expression of this gene in granulosa cells of PCOS women who received myo-Inositol in the present study probably contributes to improved oocyte competence.

Although the findings of our study provide some new molecular evidence about the possible mechanism of myo-Inositol effect on the quality of oocyte and embryo based on improved granulose cell gene expression, we did not observe an improved cumulative pregnancy rate as the final ART outcome among PCOS patients who received myo-Inositol. This might be related to the limited number of subjects in this trial, which necessitates further RCTs with larger sample size to conclude the clinical implications of our findings.

In summary, the results of our study indicate that myo-Inositol treatment may be associated with a change in the expression of some genes in granulosa cells, which may increase the rate of oocyte maturation, fertilization rate, and the quality improvement of the embryos derived from PCOS patients undergoing ART. The clinical implication of this finding should be evaluated in further RCTs.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest with the subject matter of this manuscript.

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