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The effect of myo-inositol, vitamin D3 and melatonin on the oocyte quality and pregnancy in *in vitro* fertilization: a randomized prospective controlled trial

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Abstract. – OBJECTIVE: To evaluate the efficacy of a treatment with myo-inositol (MI) plus melatonin and vitamin D3 in women underwent intra cytoplasmatic sperm injection (ICSI).

PATIENTS AND METHODS: 100 women undergoing ICSI procedure were enrolled and randomly divided 1:1 in two groups. The study group was treated with 2 g MI, 50 mg Alpha-Lactalbumin (alpha-LA) and 200 μg folic acid in powder every morning for 6 months (3 months before oocyte pick up and 3 months after ICSI); the same patients underwent treatment with 600 mg MI, 1 mg melatonin plus 200 μg folic acid during the evening for 3 months before oocyte pick up; subsequently the pick up these patients were treated with 600 mg MI, 200 μg folic acid, 1 mg melatonin, 50 μg vitamin D3 as cholecalciferol until the 12th week of gestation. The control group was treated with 200 μg folic acid twice a day.

Clinical pregnancy rate was evaluated as primary outcome, followed by blastocyst and oocyte quality, as well as gestational period as secondary outcomes.

RESULTS: Treatment significantly improved blastocyst and oocyte quality in the study group, achieving the 42% of clinical pregnancies vs. 24% in the control group, even though the course of pregnancy did not significantly differ between the groups. However, the mean gestational period was shorter in the control group.

CONCLUSIONS: The supplementation of MI in combination with melatonin in the first 3 months before oocyte pick up and with vitamin D3 in the further 3 months could represent an innovative support for all those women undergoing ICSI.

Key Words:
Myo-inositol, Melatonin, Vitamin D3, ICSI, Oocyte quality.

Introduction

Problems with achieving offspring are becoming increasingly more common in society. A decrease in fertility is conditioned by both male and female factors[1]. The main factor responsible for fertility is an appropriate quality of female and male gametes[2]. While male sperm is easy to obtain and examine, on the opposite, the quality of female oocyte is often discovered only during the *in vitro* fertilization procedure.

Many factors exert a detrimental effect on the quality of the egg cells. The most harmful associated phenomenon is oxidative stress, due to the ageing of the organism[3,4]. Furthermore, the quality of the oocyte depends on the proper course of ovarian stimulation using gonadotropins. It was demonstrated that myo-inositol (MI) contributes to the effect of gonadotropins[5]. In the mammalian female reproductive system MI levels are considerably higher than in blood serum. This indicates that undoubtedly MI plays crucial functions in the ovary. Of note, MI (as inositol 1,4,5-trisphosphate, InsP3) plays the role of one of the Follicle-stimulating hormone (FSH) second messengers, ensuring a proper oocyte maturation[6,7].

Scholars[8] conducted on mouse models suggested that MI promotes meiotic progression of oocytes into fertilization-competent eggs, whereas depletion of MI in the ovary can change the physiological process of oocyte maturation.

A plenty of evidence[9-11] reported that high MI concentrations in the follicular fluids are strongly correlated with higher oocytes quality, meanwhile low concentration of MI correlates with

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poor oocyte quality. In this regard, MI involvement in oocyte maturation is clear and it could be considered as oocyte quality marker[9-11]. Further evidence showed that MI enhances the quality of the blastocysts, whereas its stereoisomer, D-chiro-inositol (DCI), above a certain level, exerts a negative effect[12].

Furthermore, melatonin and its synthetic machinery are present in ovary and placenta[13]. In these districts, melatonin plays different roles in fertility outcomes, including oocyte quality, embryo implantation, as well as foetus development and parturition. On this regard, it was observed that melatonin exerts a direct effect on the reproductive events, such as folliculogenesis, oocyte maturation and formation of the *corpus luteum*. Studies[14,15] concerning the molecular mechanism of action clearly demonstrated a strong link between maternal melatonin secretion and embryonic and foetal development. In this regard, the supplementation with melatonin reduces oxidative stress through melatonin membrane receptors activation. Moreover, melatonin is involved in thyroid function exerting an effect on the earliest stages of pregnancy and the whole gestational period[14,15].

Also, vitamin D is deeply involved in supporting a successful pregnancy. Growing literature reports a correlation of vitamin D deficiency with male and female fertility, as well as its efficacy in improving assisted reproductive techniques (ART) outcomes[16]. Indeed, vitamin D has many physiologic effects acting simultaneously and/or in synergy with progesterone; for this reason, vitamin D was defined a steroid hormone with progesterone-like activity[16]. These two hormones cooperate each other mutually reinforcing their activity. In this regard, soon after progesterone release, vitamin D secretion increases in the occurrence of pregnancy. Calcitriol, the most active form of vitamin D, contributes to the preparation of endometrial receptivity. In addition, it supports the process of implantation and the course of pregnancy *via* pathways, which are different although similar to those, used by progesterone. As result, a significant synergy of action occurs. It is becoming increasingly clear that vitamin D is extremely important from the luteal phase onwards[16-18].

This clinical study aims to evaluate the combined effect of MI, melatonin and vitamin D3 supplementation on oocyte quality and for achieving pregnancy in the *in vitro* fertilization procedures (IVF).

Patients and Methods

Patients and Study Design

This randomized prospective controlled superiority study enrolled 100 women and was conducted from August 2018 to June 2019 in the 'Ovum Reproduction and Andrology' Non-Public Health Care Unit in Lublin (Poland).

Inclusion criteria: patients aged between 20 and 35 years old who underwent first intracytoplasmic sperm injection (ICSI) method. Only autologous cycles were considered. All women qualified for the study had ovulatory cycles and had confirmed patency of at least one fallopian tube. Partners' semen parameters were normal in accordance with WHO 2010. The study included women who previously encountered problems to get pregnant; specifically, they were patients that, in three consecutive cycles, had the growth of many follicles after monofollicular stimulation or problems due to follicles rupture, or had undergone to three unsuccessful inseminations. Exclusion criteria: presence of insulin resistance (IR) or hyperglycaemia; diagnosis of pathologic conditions causing ovulatory dysfunction (such as endometriosis, hyperprolactinaemia or hypothyroidism), or androgen excess (such as adrenal hyperplasia or Cushing's syndrome); diagnosis of Polycystic Ovary Syndrome (PCOS) according to Rotterdam ESHRE-ASRM Sponsored PCOS consensus workshop group; other causes for the infertility (male factor, tubal factor, etc.); intake of hormones or drugs that could potentially influence ovulation, as well as intake of supplements containing Inositols, melatonin and/or vitamin D3 in the previous 3 months before the enrolment.

This study was conducted following the Ethical principles of the Declaration of Helsinki and the national laws. The 100 women enrolled in the study were informed through written consent and randomized 1:1 in two groups (study group and placebo group). All patients in study group (n= 50) were treated with 2 g MI, 50 mg alpha-Lactalbumin (alpha-LA) and 200 µg folic acid (Inofolic HP®, LoLi Pharma sachets) every morning for 6 months (3 months before oocyte pick up and 3 months after ICSI); the same patients in the evening underwent the treatment with 600 mg MI, 1 mg melatonin plus 200 µg folic acid (Inofolic Plus®, LoLi Pharma soft capsules, Rome, Italy) during the evening for 3 months before oocyte pick up. Subsequently the oocyte picks up these patients had a change in the evening administration. They were treated with 600 mg MI, 200 µg folic acid, 1 mg melatonin, 50 µg equivalent to 2000

Table I. Treatment schedule.

Patients	Number patients	Treatment for 3 months	Oocyte pick up	Treatment for 3 months (only if pregnant)
Study group	50	**Inofolic HP** (morning) **Inofolic Plus** (evening)	X	**Inofolic HP** (morning) Inofolic Luteal (evening)
Control group	50	Placebo	X	Placebo

IU vitamin D3 as cholecalciferol (Inofolic Luteal®, LoLi Pharma soft capsules) until the 12th week of gestation. In the following days only the pregnant women continued with the new schedule of treatment. The patients undergoing standard IVF treatment carried out in the Centre were considered as control (placebo) group (50 patients) and were treated with 200 µg folic acid twice a day. The treatment schedule is reported in the Table I.

As primary outcome, the clinical pregnancy rate was considered. The total recombinant FSH (rFSH) units, the number of ovarian stimulation days, the endometrial thickness, the estradiol (E2) levels, the number of retrieved oocytes, as well as their quality, the blastocyst quality and gestational period were evaluated as secondary outcomes.

Ovarian stimulation was performed by administrating luteal gonadotropin-releasing hormone analogue (GnRHa) (Gonapeptyl Daily®: Ferring GMBH, Kiel, Germany), and subsequently rFSH (Bemfola®: Gedeon Richter, Budapest, Hungary) in a short protocol from day 3 of the cycle. During ovulation stimulation, the number of follicles (≥17 mm) and endometrial thickness (mm) were evaluated by the Voluson E8 Expert. A morning blood sample (5 mL) was obtained on the day of recombinant human chorionic gonadotropin (rhCG) administration, and serum E2 levels (pg/mL) assessed. On day 3 of the cycle preceding ovulation, the Anti-Müllerian Hormone (AMH) (ng/mL) level was assessed in an authorized laboratory.

Thirty-six hours after administration of rhCG (Ovitrelle®: Merc-Serono, Darmstadt, Germany), vaginal ultrasound-guided aspiration of the oocyte-cumulus complexes was performed.

Oocyte denudation and ICSI were performed 3 hours after retrieval. The *in vitro* culture was carried out in 25 µL of Cleavage medium (COOK, Medical Sydney IVF, Australia) under mineral oil until day 2 (2-5 cells stage) in automated incubators with 5% CO_2 at 37°C.

Fifty hours from ICSI the *in vitro* culture media was changed to blastocyst medium (COOK, Sydney IVF, Australia). The study was approved by the Bioethics Commission.

Quality of Oocytes

Oocyte quality was described by 6 characteristics: each of which was assigned a value of -1 (worst), 0 (average) or +1 (best), thereby establishing an average total oocyte score (TOS): morphology, cytoplasm[19-26], perivitelline space (PVS)[27,28], zona pellucida (ZP)[29,30], polar body (PB)[31,32] and oocyte size[33]. Only ICSI cycles and patients undergoing embryo transfer on day-5 (blastocyst) were evaluated.

Blastocyst Quality

Based on blastocyst parameters, the expansion, trophectoderm (TE) and inner cell mass (ICM) quality of the blastocysts were assessed, and the quality of embryos differentiated as 'excellent', 'good', 'average', and 'poor'.

Initially, the embryos were assigned a numeric score 1-6, based on the degree of expansion, and hatching status: (1) early blastocyst-blastocoel formed less than 50% of the volume of the embryo; (2) blastocyst-blastocoel formed more than 50% of the volume of the embryo; (3) full blastocyst-blastocoel completely filling the embryo; (4) expanded blastocyst-blastocoel volume was larger than that of the early embryo, accompanied by the thinning of the zona; (5) hatching blastocyst-trophectoderm started to herniate through the surrounding zona; and (6) hatched blastocyst-blastocyst completely escaped from the zona. Subsequently, the development of the ICM and TE were assessed for blastocysts graded 3-6 (full blastocysts onward). The ICM grade was determined as follows: A, tightly packed, with many cells; B, loosely grouped, with several cells; and C, very few cells. The TE grades consisted of A, many cells, forming a cohesive epithelium; B, few cells, forming a loose epithelium; and C, very few large cells[34].

In order to compare the results of the present study and those by Capalbo et al[35] and Irani et

al[36] the blastocysts were assigned a three-character score according to their degree of blastocyst expansion, as well as ICM and TE grades, immediately prior to biopsy.

According to the score, the blastocytes were divided as follows:

1. "excellent" (≥3AA)
2. "good" (3-6AB, 3-6BA, 1-2AA)
3. "average" (3-6BB, 3-6AC, 3-6CA, 1-2AB, 1-2BA)
4. "poor" (1-6BC, 1-6CB, 1-6CC, 1-2BB).

Two weeks after the punction, the hCG level was assessed to confirm whether the patient was pregnant. At week 6 of pregnancy, embryonic cardiac activity was assessed using ultrasound.

Statistical Analysis

Statistical analysis of the data was performed using the statistical software package SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA). Arithmetic means (M) and standard deviations (SD) were estimated for continuous variables or absolute numbers (n) and percentages (%) for categorical variables.

Student's *t*-test for two independent samples was used to compare continuous variables or chi-square test to compare categorical variables between the study group and the control group.

A significance level of $p \leq 0.05$ was assumed.

Results

The mean age (A-31, B-31.2), BMI (A-24.76, B-25.11), AMH (A-3.08, B-2.87) and the number of follicles obtained during stimulation (A-8.32, BB-7.68) did not significantly differ between the two groups. As a result of ovulation stimulation, a significantly higher number of oocytes was obtained in the study group (7.88), compared to the control (6.10). In addition, a lower dose of rFSH (151.50 IU/L *vs.* 160.50 IU/L), as well as less days of stimulation (8.8 days *vs.* 10.36 days) was used for the patients in the study group. The level of E2 was also higher in the supplemented women (2392.64 pg/mL) than control (1534.04 pg/mL).

The endometrium obtained as a result of stimulation, measured on the day of rhCG administration, was thicker in the study group (12.4 mm), compared to the control group (9.89 mm). All the data are showed in the Table II.

The treated group reported a significant higher oocyte quality (65.38%) than control group (18%) (Figure 1). Similarly, good blastocysts quality had a higher frequency (70%) in the study group, compared to the control group (21.28%) (Figure 1).

The treatment allowed obtaining 42% of clinical pregnancies in the study group *vs.* 24% in the control group (p=0.0498) (Figure 1). The course of pregnancy did not significantly differ between the groups (Table II); however, the mean gestational period was shorter in the control group (37.33 hbd) than in the study group (38.86 hbd).

Discussion

The study showed that the supplementation with MI, melatonin and vitamin D3 improves the effectiveness of the ICSI procedure. There

Table II. Correlation between the expression level of OIP5-AS1 and clinical characteristics of OC patients (n=52).

Variable	Study group		Control group		*p*-value
	Mean	SD	Mean	SD	
Age (years)	31.00	3.11	31.20	3.03	0.7454
BMI	24.76	2.94	25.11	2.39	0.5348
rFSH (units)	151.50	10.61	160.50	26.29	0.0270
Days (number)	8.80	1.18	10.36	1.77	<.0001
Endometrial thickness (mm)	12.40	1.22	9.89	1.11	<.0001
Follicles (number)	8.32	2.77	7.68	3.10	0.2793
AMH (ng/mL)	3.08	0.93	2.97	0.86	0.5354
E2 (pg/mL)	2392.64	1534.04	1525.34	1048.96	0.0013
Number of oocytes	7.88	2.68	6.10	2.40	0.0007
Delivery (weeks)	38.86	1.39	37.33	1.78	0.0193

Endometrium thickness, number of follicles and E2 levels were evaluated two days before puncture. Oocytes were counted on the day of puncture. AMH levels were assayed one month before the start of stimulation.

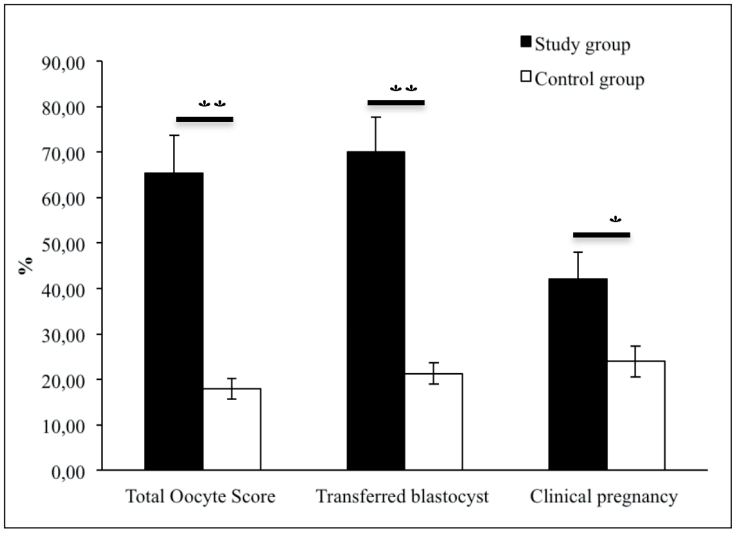

Figure 1. Comparison between study and control groups at the end of treatments. Differences were considered significant for $p<0.001$ (***) and $p\leq0.05$ (*).

are no previous reports in literature concerning the supplementation of this posology scheme. On the other hand, the available scientific studies[34-37] confirm a beneficial effect of these substances on the achievement of pregnancy when applied in other combinations. In particular, MI and melatonin three months before the oocyte pick up, followed by MI and vitamin D3 for further three months demonstrated the beneficial synergic effect of these three molecules. In fact, the study group showed a significant higher oocyte and embryo quality compared to control. This beneficial effect at ovarian level is also reported in different studies about the use of melatonin, alone or in combination with MI. Moreover, data clearly report a reduced risk of Ovarian Hyper Stimulation Syndrome (OHSS) in patients underwent the treatment through a reduction of rFSH units and days of stimulation as well as a higher E2 level. It is also necessary to highlight that our results agree with Ciotta et al[38] results. As a consequence, such findings deserve to be carefully considered if we keep in mind the reduction of costs and time spent for this procedure.

We have to point out the specific therapeutic activities due to the different treatments. The combination between MI and melatonin exerted its effects on the oocytes retrieved and all the parameters related to ICSI technique, whereas Vitamin D plus MI and melatonin were responsible for the increase in pregnancy rate (the primary outcome of our study). Therefore, the differentiated administration of MI and melatonin before ICSI, and MI plus melatonin and Vitamin D after ICSI demonstrated to be useful since the three molecules collaborated in productive way for improving the results of this IVF technique.

Pacchiarotti et al[39], in their study tested the synergistic effect of MI and melatonin in IVF protocols with PCOS patients in a randomized, controlled, double-blind trial. In their study, 526 PCOS women were divided into 3 groups and treated daily as follows: Controls (only folic acid 400 µg), Group A (MI 4000 mg, folic acid 400 µg and melatonin 3 mg), and Group B (MI 4000 mg and folic acid 400 µg). Worth of note, the treated group A reported a beneficial effect on the oocyte and embryo quality[39] similar to that observed in the present study.

Unfer et al[40] evaluated pregnancy outcomes after the administration of MI combined with melatonin in women who failed to conceive in previous IVF cycles due to poor oocyte quality. They treated 46 women with 2 g MI plus 200 µg folic acid in the morning and 2 g MI, 200 µg folic acid and 3 mg melatonin in the evening, and then, proceeded to a new IVF cycle. After this treatment, the number of mature oocytes, fertilization rate, number of both, total and top-quality embryos transferred, were statistically higher compared to the previous IVF cycle, while there was no difference in the number of retrieved oocytes. After the treatment, a total of 13 pregnancies occurred, 9 of them were confirmed echographically; 4 evolved in spontaneous abortion. These observations[40] are similar to those in the present study.

In the clinical trial by Rizzo et al[41], 65 women undergoing IVF cycles, from the day of GnRH administration, were randomized in two groups to receive 2 g MI, 200 µg folic acid, and 3 mg

melatonin (32 women, group A), or the same amount of MI plus folic acid without melatonin (33 women, group B), twice a day.

The researchers found out that the mean number of oocytes retrieved did not differ between the groups (7.88 +/- 1.76 *vs.* 7.67 +/- 1.88; p=0.65). However, the group co-treated with melatonin reported a significantly larger mean number of mature oocytes (6.56 +/- 1.64 *vs.* 5.76 +/- 1.56; p= 0.047) and a lower mean number of immature oocytes (1.31 +/- 0.74 *vs.* 1.91 +/- 0.68; p= 0.001). The mean number of embryos of top-quality (class 1 and 2) proved to be higher in group A (1.69 +/- 0.64 *vs.* 1.24 +/- 0.75; p= 0.01).

Papaleo et al[42] studied 30 participants, from the day of GnRH administration, who received 2 g MI plus 200 µg folic acid twice a day, and 30 controls who received folic acid alone (same amount). It was found that total rFSH units (1.958 +/- 695 *vs.* 2,383 +/- 578) and number of days of stimulation (11.4 +/- 0.9 *vs.* 12.4 +/- 1.4) were significantly reduced in the MI-treated group, which is consistent with the results of the present study. It was also found that the mean number of oocytes retrieved did not differ in the two groups. However, in the group treated with MI the mean number of germinal vesicles and degenerated oocytes was significantly reduced (1.0 +/- 0.9 *vs.* 1.6 +/- 1.0), with a trend for increased percentage of oocytes in metaphase II (0.82 +/- 0.11% *vs.* 0.75 +/- 0.15%). The results obtained by both Rizzo et al[41] and Papaleo et al[42] confirmed our results.

Another important aspect of our trial is that the treated patients showed a thicker endometrium with respect to the control group. This result could explain the significant higher number of clinical pregnancies. We may speculate that the endometrium of patients treated with MI in combination with melatonin and, subsequently, with vitamin D3 has a higher receptivity. This evidence can be supported by the theory that melatonin plays a role in endometrium proliferation, whereas vitamin D3, acting as progesterone-like, might cooperate with this hormone in supporting pregnancy. On this regard, further studies are needed for achieving stronger evidence.

Similar data about vitamin D was detected by Fatemi et al[43] concerning the effect of vitamin D on the effectiveness of ICSI. In this trial 105 PCOS infertile women scheduled for ICSI were enrolled in a double-blinded randomized placebo-controlled trial to receive daily 400 mg alpha tocopheryl and 3300 IU/L vitamin D3 (n=52) or placebo (n=53) for 8 weeks. The authors reported that clinical pregnancy and implantation rate were significantly higher in the treatment group. The study also showed that there is a positive weak relationship between vitamin D3 level, implantation rate (p=0.015), and increased clinical pregnancy (p=0.037).

Rudick et al[44] carried out a retrospective cohort study in an academic tertiary care centre, among 188 infertile women undergoing IVF. Vitamin D status was measured by assessing circulating levels of 25(OH)D in frozen, never previously thawed serum samples using radioimmunoassay (RIA). Intra-and interassay coefficients of variation were 10.5 and 8.2%, respectively. Serum 25(OH)D was categorized according to clinically accepted ranges for vitamin D deficiency (<20 ng/ml), insufficiency (20-30 ng/ml) and replete (>30 ng/ml).

The relationship between vitamin D status and pregnancy rates differed by race (p<0.01). Among non-Hispanic whites, pregnancy rates declined with progressively lower levels of vitamin D, while in Asians, the reverse was found. Adjusting for age and number and quality of embryos transferred among non-Hispanic whites, the odds of pregnancy were 4 times higher in vitamin D replete *vs* deficient patients. Live birth rates reflected pregnancy rates. Vitamin D status was not associated with ovarian stimulation parameters or with markers of embryo quality. The participants of the present study belonged to the non-Hispanic whites, which explain similarity of the results obtained.

The results obtained by different researchers and those of the present study confirm that it is justified to administer the combination of MI, melatonin and vitamin D3 to women undergoing infertility therapy.

Conclusions

Summarizing, the supplementation of MI plus melatonin in the first 3 months before oocyte pick up and MI plus melatonin and vitamin D3 in the further 3 months could represent an innovative support for all those women undergoing ICSI.

Indeed, our treatment showed several clinical benefits, including better fertilization and pregnancy outcomes as well as reduced risk of OHSS.

Undoubtedly, even though these results are very encouraging, further investigations with larger cohort of patients are needed.

Conflict of Interests

The authors declare that they have no conflict of interest.

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