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Effect of the treatment with myo-inositol plus folic acid plus melatonin in comparison with a treatment with myo-inositol plus folic acid on oocyte quality and pregnancy outcome in IVF cycles. A prospective, clinical trial

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Abstract. – *Objective:* **The aim of the study was to evaluate the efficacy of a treatment with myo-inositol plus folic acid plus melatonin compared with myo-inositol plus folic acid alone on oocyte quality in women underwent in vitro fertilization (IVF) cycles.**

Design: **A prospective, clinical trial.**

Materials and Methods: **Starting on the day of GnRH administration, 65 women undergoing IVF cycles were randomized in two groups to receive myo-inositol plus folic acid plus melatonin (32 women, group A), and myo-inositol plus folic acid (33 women, group B), administered continuously.**

Primary endpoints were number of morphologically mature oocytes retrieved (MII oocytes), embryo quality, and pregnancy rate. Secondary endpoints were the total number of oocytes retrieved (immature and mature oocytes), fertilization rate per number of retrieved oocytes and embryo cleavage rate.

Results: **The mean number of oocytes retrieved did not differ between the two groups (7.88 ± 1.76 vs 7.67 ± 1.88; *P*=0.65). Whereas the group cotreated with melatonin reported a significantly greater 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 embyos of top-quality (class 1 and 2) resulted higher in the group A (1.69 ± 0.64 vs 1.24 ± 0.75; *P*=0.01). Fertilization rate did not differ between the two groups. A total of 22 pregnancies were obtained (13 in group A and 9 in group B; *P*=0.26). Clinical pregnancy rate and implantation rate were in tendency higher in the group cotreated with melatonin, although the differences did not reach statistical significance. Biochemical pregnancy rate and abortion rate were similar in both groups.**

Conclusion: **melatonin ameliorates the activity of myo-inositol and folic acid by improving oocyte quality and pregnancy outcome in women with low oocyte quality history.**

Key Words:

Myo-inositol, Melatonin, IVF cycles.

Introduction

Many subfertile couples, who failed to conceive naturally, seek help by means of artificial reproduction techniques such as in vitro fertilization (IVF) to achieve pregnancy. Although these techniques have improved the treatment of subfertile couples, the chance of achieving a clinical pregnancy remains around 25% per started cycle[1].

The quality of oocytes plays a key role in the development of a clinical pregnancy. In humans, in fact oocytes of poor quality may be the cause of women infertility and an important obstacle in successful i*n vitro* fertilization (IVF)[2].

The competence of oocytes depends on numerous processes taking place during the whole oogenesis, but its final steps such as oocyte maturation, seem to be of key importance. Follicular fluid (FF) provides a very important microenvironment for the development of oocytes. FF may be regarded as a biological "window" reflecting metabolic and hormonal processes occurring in the microenvironment of the maturing oocyte before ovulation and also as a predictor of outcome parameters such as fertilization, embryo cleavage and pregnancy rates in IVF[3]. It is reasonable to think that some biochemical characteristics of the FF surrounding the oocyte may play a critical

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role in determining oocyte quality and the subsequent potential to achieve fertilization and embryo development. For example, mild to moderate intrafollicular hyperhomocysteinemia is associated with detrimental effects on reproductive outcome, ranging from congenital malformations and miscarriages to pregnancy induced hypertension and low birthweight[4-6]. The main causes of hyperhomocysteinemia include a dysbalance between the intake of folate, cobalamin, pyridoxine and methionine, metabolic derangements and related genetic variations[7]. It has been demonstrated that a preconception folic acid treatment positively affects the microenvironment of the maturing oocyte in humans, by decreasing total homocysteine concentrations in pooled follicular fluid and increasing follicle diameter[8].

Another parameter taken into account in terms of oocyte quality in previous studies was the concentration of myo-inositol in FF[9,10]. Myo-inositol is an isoform of inositol and belongs to the vitamin B complex. Myo-inositol (MI) is widely distributed in nature. Considering all the results coming out from previous studies[11-13] it is clear that myo-inositol is a precursor of the synthesis of phosphoinosides, in particular it constitutes the phosphatidylinositol signal transduction system, known to be involved in the regulation of several cellular function, including cell proliferation[14].

The presence of MI in human body fluid, its role as precursor of the inositol phospholipids responsible for the generation of important intracellular signals essential for mammalian oocyte development[15,16] and its higher concentration in FF containing oocytes of good quality[17] suggestes that the supplementation with myo-inositol in IVF techniques, could positively influence the final result of the reproduction technique. In a previous study[18] the efficacy of a periconceptional treatment with myo-inositol plus folic acid on oocyte quality and pregnancy rate comparing with a treatment with folic acid alone has been evaluated. It has been demonstrated that the treatment has improved the number of oocytes of good quality.

Reactive oxygen species (ROS) has been considered to play a critical role in the success of different ARTs[19]. ROS are produced within the follicle, especially during the ovulatory process[20], and it is believed that oxidative stress may be a cause of poor oocyte quality[21]. High levels of oxidants, as H_2O_2, as been found in fragmented embryos[22].

There is evidence that melatonin plays an important role in the regulation of reproductive activity[23] with some potential effects in humans as well[24]. High levels of melatonin[25] have been found in human preovulatory follicular fluid in concentrations which are almost three-fold higher than serum levels[26,27]. At the present time, the physiological role of intrafollicular melatonin is not fully understood. Melatonin as well as its metabolites are potent direct free radical scavengers[28-31] and indirect antioxidants by virtue of their ability to modulate gene transcription for antioxidant enzymes[32]. The antioxidant properties of melatonin have been extensively studied and the use of this molecule as a cell protector and as a potential disease preventing agent has been summarized[33-37]. Furthermore, the wide action of melatonin on the ovary functions has been illustrated[38]. In a recent study the role of melatonin in protecting oocytes from ROS damage and consequently in improving oocyte quality and pregnancy rate has been detected[39].

Considering the proven positive effects of the supplementation of myo-inositol plus folic acid on the FF composition and consequently on the oocyte quality, the current study was undertaken to examine whether the addition of melatonin improves more oocyte quality and pregnancy rate in comparison with the administration of myo-inositol plus folic acid alone, in women undergoing IVF cycles.

Materials and Methods

Patients

Sixty-five women, aged between 35 and 42, with infertility factors reporting for IVF by controlled ovarian stimulation (long protocol) were included. For the purposes of this study, infertility factors refer to low oocyte quality detected in the previous IVF cycles.

According to a randomization table, patients were assigned to receive either 2 g myo-inositol twice a day combined with 200 mg folic acid and 3 mg melatonin (*n*=32, group A; Inofolic *plus*, LO.LI.pharma Srl, Rome, Italy), or 2 g myo-inositol twice a day combined with 200 mg folic acid (*n*=33, group B; Inofolic, LO.LI.pharma Srl), administrated continuously from the day of GnRH administration. The Institutional Review Board approved the protocol, and all patients gave a written informed consent before entering in the study.

Follicle Stimulation Protocol

All the women enrolled were down-regulated with a GnRH agonist (Decapeptyl; Ipsen, Milan, Italy) from mid-luteal phase onwards and, when optimally down-regulated, were stimulated with recombinant FSH (Gonal F; Serono, Rome, Italy) with a starting dose of 150 IU/die. The FSH dose was adjusted according to the individual response. Follicular size was monitored regularly by ultrasound and serum estradiol assays. Intramuscolar hCG was administered when average diameter of the leading follicles reached at least 18 mm. Cycle was cancelled if E_2 level was >4,000 pg/mL, because of high risk for ovarian hyperstimulation syndrome.

IVF Procedure

Oocyte retrieval was performed 36 hours after hCG injection with transvaginal guidance. Cumulus and corona radiata cells were immediately removed after retrieval by a short exposure to HEPES-buffered medium (Quinn's Advantage Hepes Medium; Sage IVF, Trumbull, CT, USA) containing 20 IU/mL hyaluronidase (Sage IVF) and gentle aspiration in and out of a Pasteur pipette and mechanically cleaned from the remaining surrounding cumulus cells by aspiration using a denuding pipette (Denuding Flexi-Pet; Cook, Australia) with a 170-130 μL diameter. The denuded oocytes were then assessed for their meiotic maturation status[40]. In preparation for IVF, oocytes with an extruded first polar body presumably at the metaphase II stage (MII) were selected. According to Italian IVF law a maximum of three oocytes per patient were inseminated and spare MII oocytes were cryopreserved, if required[41].

Luteal Phase

On the day of ovum pick-up, intramuscular progesterone administration, 50 mg daily, was started and treatment was continued until either a serum pregnancy test result was negative or an embryonic heart beat was sonographically confirmed.

Embryo Quality Classification

The embryos were classified according to the criteria proposed by Steer[42], as follows: grade 1, equally-sized blastomeres with no fragmentation; grade 2, equally- or unequally-sized blastomeres with <20% overall fragmentation; grade 3, equally- or unequally-sized blastomeres with 20%-50% fragmentation; and grade 4, equally- or unequally-sized blastomeres with >50% fragmentation. The embryos with <20% overall fragmentation (grade 1 or 2), together with >6 blastomeres on day 3 were considered as good embryos[43]. Embryo quality was assessed before the transfer that occurred in all patients approximately 48 h (4-cell stage) after insemination.

Determination of Pregnancy States

A small and transitory increase in β-hCG levels was defined as a biochemical pregnancy. A clinical pregnancy was determined by the visualization of an embryo with cardiac activity at 6-7 weeks of pregnancy. Spontaneous abortion was classified as the loss of the pregnancy between the fifth and twelfth weeks of gestation.

Statistical Analysis

We compared the baseline characteristics of the women, ovum pick-up and insemination outcomes using χ^2 analysis for discrete variables and unpaired Student *t* test for continuous variables. Frequency data (pregnancy outcomes) were compared using χ^2 analysis or Fisher exact test. *P*-values <0.05 were taken as statistically significant. SPSS statistical package for Windows, version 15.0 was used.

Results

During the study period, 65 patients conforming to the inclusion criteria were randomized into two groups: Group A (myo-inositol plus folic acid plus melatonin) consisted of 32 patients and group B (myo-inositol and folic acid) consisted of 33 patients. Mean patient age, body mass index (BMI), duration and cause of infertility were similar in the two groups (Table I).

Estradiol on the day of hCG injection was 2,313.31 ± 1,241.30 versus 2,201.74 ± 1,212.26 nmol/l, respectively, with no significant difference between the groups. Progesterone on the day of hCG was 1.51 ± 1.28 vs 1.58 ± 0.98 respectively, with no significant difference between the groups (Table I).

The mean number of oocytes retrieved did not differ between the two groups (7.88 ± 1.76 vs 7.67 ± 1.88; *P*=0.65). Whereas the group cotreated with melatonin reported a significantly greater mean number of mature oocytes (MII oocytes: 6.56 ± 1.64 vs 5.76 ± 1.56; *P*=0,047) and a lower mean number of immature oocytes (germinal

Table I. Demographic characteristics, infertility status and hormonal outcomes of patients treated with Inofolic plus (Group A; n=32) or Inofolic (Group B; n=33).

Patient characteristics	Group A (Inofolic plus) (n = 32)	Group B (Inofolic) (n = 33)	p
Patient age (yrs)	37.81 ± 2.61	38.09 ± 1.97	NS
Duration of infertility (yrs)	4.18 ± 1.35	3.99 ± 1.39	NS
Body mass index (kg/m^2)	26.65 ± 2.79	27.45 ± 2.18	NS
Indication for 1° IVF treatment			
Tubal factor	6	7	NS
Endometriosis	8	9	NS
Ovulatory factor	8	8	NS
Unexplained	9	7	NS
Other	1	2	NS
Estradiol (pg/mL) on hCG day	2,313.31 ± 1,241.30	2,201.74 ± 1,212.26	NS
Progesterone (ng/mL) on hCG day	1.51 ± 1.28	1.58 ± 0.98	NS

Data are presented as mean ± SD. NS = no statistically significant; hCG = human chorionic gonadotropin.

vesicles and degenerated oocytes: 1.31 ± 0.74 versus 1.90 ± 0.68; *P*=0.001; Table II).

The oocytes inseminated (not more than 3 per patient, in compliance with Italian IVF law) generated embryos classified in four classes due to their quality[42,43]. The mean number of embyos of top-quality (class 1 and 2) resulted higher in the group A (1.69 ± 0.64 versus 1.24 ± 0.75; *P*=0.01), even though the fertilization rate and cleavage rate did not differ between the two groups (0.82 ± 0,19 versus 0.79 ± 0,23; *P*=0.51 and 0.89 ± 0,18 vs 0.87 ± 0,23; *P*=0.67, respectively; Table II).

A total of 22 pregnancies were obtained (13 in group A and 9 in group B; *P*=0.26; Table III). Clinical pregnancy rate and implantation rate was in tendency higher in the group cotreated with melatonin (*P*=0.25 and *P*=0.56 respectively), although these differences did not reach statistical significance. Biochemical pregnancy rate and abortion rate were similar in both groups (Table III).

Discussion

In routine IVF cycles, it is common to see that few morphologically mature oocytes (MII oocytes) obtained from matured follicles remain unfertilized or, if fertilized, result in poor-quality embryo formation, under the same culture conditions. Follicular fluid makes up the actual environment of the mature oocyte before fertilization

Table II. Oocyte maturity and embryo score in patients who received Inofolic plus (group A; n=32) or Inofolic (group B; n=33).

Variable	Group A (Inofolic plus) (n = 32)	Group B (Inofolic) (n = 33)	p
No. of oocytes retrieved	7.88 ± 1.76	7.67 ± 1.88	NS
No. of mature oocytes (MII)	6.56 ± 1.64	5.76 ± 1.56	0.047
No. of immature oocytes (GV, degenerated oocytes)	1.31 ± 0.74	1.90 ± 0.68	0.001
Fertilization rate	0.82 ± 0.19	0.79 ± 0,23	NS
Cleavage rate	0.89 ± 0.18	0.87 ± 0,23	NS
No. of embryos transferred	2.03 ± 0.69	1.91 ± 0.58	NS
No. of top-quality embryos transferred (score 1 and 2)	1.69 ± 0.64	1.24 ± 0.75	0.01
Embryo score grade 1	0.72 ± 0.46	0.63 ± 0.50	NS
Embryo score grade 2	1.13 ± 0.49	0.94 ± 0.61	NS
Embryo score grade 3	0.50 ± 0.51	0.58 ± 0.61	NS
Embryo score grade 4	0.03 ± 0.18	0.27 ± 0.52	0.01

Data are presented as mean ± SD. NS = no statistically significant; MII = Metaphase II; GV = germinal vesicle.

Table III. Pregnancy outcomes of patients who received Inofolic plus (group A; n=32) or Inofolic (group B; n=33).

Variable	Group A (Inofolic plus) (n = 32)	Group B (Inofolic) (n = 33)	p
Pregnancies, n	13	9	NS
Biochemical pregnancies, n (%)	1 (7.7)	1 (1.1)	NS
Clinical pregnancies b, n (%)	12 (37.5)	8 (24.2)	NS
Implantation rate a, %	19.3	16.2	NS
Spontaneous abortion, n (%)	2 (16.7)	2 (25.0)	NS

NS = no statistically significant. [a]Total number of embryos transferred. [b]Number of cycles.

and may influence IVF outcome parameters such as fertilization, embryo cleavage and pregnancy rates[44]. This environment, in addition to granulosa cells, growth factors and steroids hormones, contains leukocytes, cytokines and macrophages, all of which can produce ROS[45]. Hence, ROS may be produced by either the environment or impaired metabolism of the oocyte, or both.

Melatonin (*N*-acetyl-5-methoxytryptamine) is a small lipophilic indoleamine generated primarily in pineal gland and secreted in a circadian manner with high levels occurring in all species at night. Melatonin plays a key role in a variety of important physiological functions, including in the seasonal timing of reproductive activities in a number of mammalian species[24,46,47]. In particular, it has been shown to have a direct effect on the female reproductive tract, where it regulates sex steroid secretion[48,49].

The present study is the first trial focusing on this molecule combining with two of the most important molecules that show to improve oocyte quality: myo-inositol and folic acid. The data showed that in patients undergoing IVF that have reported low oocyte quality in previous cycles, the treatment with melatonin plus myo-inositol and folic acid, compared with myo-inositol plus folic acid alone, reduced the number of germinal vesicles and degenerated oocytes and enhanced the number of morphologically mature oocytes at ovum pick-up without compromising the total number of retrieved oocytes. An important result is also the greater number of top-quality embryos in the group cotreated with melatonin. The number of total pregnancies registered is higher in patients cotreated with melatonin but the results are not statistically significant perhaps because the little number of patients recruited. These results are in line with other studies, suggesting the positive effect that melatonin plays on oocyte quality and pregnancy outcome[39].

In conclusion, these observations suggest that melatonin ameliorates the activity of myo-inositol and folic acid by improving oocyte quality and pregnancy outcome in patients with low oocyte quality history. Further investigations on greater population could be interesting to confirm these positive data.

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