

# The Boosting Effects of Melatonin on the In Vitro Fertilization (IVF) of Women with Polycystic Ovary Syndrome

Shamim Pilehvari<sup>1</sup>, Mahnaz Yavangui<sup>2</sup>, Elham Paknahad<sup>1</sup>, Zahra Cheraghi<sup>3</sup>, and Marzieh Ghorbani<sup>4,\*</sup>

<sup>1</sup>Clinical Research Development Unit of Fatemeh Hospital, Hamadan University of Medical Sciences, <sup>2</sup>Endometrium and Endometriosis Research Center, Hamadan University of Medical Sciences, <sup>3</sup>Modeling of Non-communicable Diseases Research Center, Hamadan University of Medical Sciences, <sup>4</sup>Department of Clinical Biochemistry, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

Polycystic ovary syndrome (PCOS) is one of the most critical disorders, which affects approximately 20% of women of childbearing age and melatonin supplementation in these women can be effective. However, human studies in this area are particularly limited to IVF candidates. The aim of this clinical trial study was to evaluate the effect of melatonin on the in vitro fertilization (IVF) in PCOS involved women. In this clinical trial study, a total of 320 women with PCOS were randomly assigned to the intervention and control groups. Patients in the intervention group (n=160) received a combination of melatonin and metformin (3 mg and 500 mg, respectively) three times a day. The control group (n=160) received metformin 500 mg from the luteal phase of the cycle before the start of gonadotropin. Oocyte and embryo quality, number of oocytes, and pregnancy outcomes were compared in both groups. Our study revealed that the frequency of Metaphase II oocytes (69.9% vs. 57.9%,  $p < 0.001$ ) and the number of embryos of the top-quality (grade A) were higher in the group treated with melatonin (40.3% vs. 29.9%,  $p = 0.001$ ). The rate of clinical pregnancy and implantation were also higher in the intervention group. The odds of clinical pregnancy in the intervention group was 1.8 times ( $p = 0.039$ ). Moreover, oral melatonin supplementation was effective in patients with PCOS, who were candidates for IVF because of the increased quality of mature oocytes, top-quality embryos, and increased odds of clinical pregnancy.

**Key Words:** Polycystic Ovary Syndrome; Melatonin; Assisted Reproductive Techniques

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## INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most important disorders in the women of reproductive age, whose main features are hyperandrogenism, polycystic ovarian morphology, irregular menstrual cycles, hirsutism, and chronic anovulation. The National Institutes of Health (NIH) defines it as “hyperandrogenism with ovulatory disorder.” In the reproductive stage of women, the occurrence rate of PCOS is 5-10% and its prevalence is about 12-20%. In addition, these patients experience varying degrees of metabolic and endocrine disorders and increased inflammation. These characteristics lead to poor oocyte quality and even

infertility in 74% of these patients. The causes of PCOS are not fully understood and several epigenetic and environmental factors may be impressive in the development of this disorder; one of the them is oxidative stress (OS).<sup>1</sup>

Free radicals and antioxidants affect the ovarian folliculogenesis regulation (progress of meiosis II), secretion of gonadotropins, DNA damage, and progressing luteal phase of the ovary.<sup>2</sup> That's why every month with the growth of a group of follicles in the ovary only one of them becomes the dominant follicle; this process is controlled by the increased level of ROS and inhibited by antioxidants. Furthermore, in the follicular fluids of PCOS patients, there is a decrease in the concentration of antioxidants, which leads to the disruption of the cycle of follicular and luteal

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## Corresponding Author:

Marzieh Ghorbani  
Department of Clinical Biochemistry,  
Faculty of Medicine, Hamadan  
University of Medical Sciences,  
Shahid Fahmideh Street,  
Hamadan 6517838695, Iran  
Tel: +98-8138382110  
Fax: +98-8138382112  
E-mail: Rozhinaghorbani@yahoo.com

phases of the ovary.<sup>3-5</sup> Antioxidants decrement, as well as an increased level of ROS directly is related to the reduced oocyte maturation and fertilization rates, poor embryo quality, and reduced pregnancy rates.<sup>5</sup> Therefore, reducing oxidative stress in PCOS patients is considered as a therapeutic strategy.

One of the most effective substances that reduce oxidative stress is melatonin. Melatonin (N-acetyl-5-methoxy-tryptamine) is an indole amine hormone derived from the essential amino acid tryptophan. It is a multifunctional molecule synthesized and release by the pineal gland in response to darkness. It is also produced in many tissues, such as the ovaries, testes, uterus, placenta and skin. It plays a key role in a variety of important physiological functions, such as circadian rhythms, reproduction, inhibition of free radicals; it has also anti-inflammatory and anti-apoptotic roles.<sup>6-9</sup> Melatonin performs its antioxidant function in the target cells through the MT1 and MT2 receptors. They are the members of the transmembrane G protein-coupled receptor family and act on the protein kinase activity and leading to decrease of cAMP and cGMP cyclase and increase of levels of inositol triphosphate (IP3) and 1,2-diacylglycerol (DAG).<sup>10</sup> Melatonin is not only a hormone but also a cell protector. Melatonin has also some impressive roles in the reproductive system; it affects oocyte maturation, increases fetal growth and even ovarian aging, and also neutralizes the germ cell-free radicals.<sup>11-13</sup> It has been implicated in many processes including oxidative stress, immune modulation<sup>14</sup> and regulation of arterial blood pressure in type 2 diabetic patients.<sup>15</sup> It also acts on the pancreatic b-cells and improves insulin resistance.<sup>16-18</sup>

Despite common fertility treatments that are used to treat infertility, there are still challenges in PCOS patients that are not well treated. This is because poor fertilization and low quality of oocytes and embryos have a negative effect on the clinical results in PCOS patients under Assisted Reproductive outcomes Technology (ART) treatment. Thus, one of the ways to increase the chances of success in fertility treatment patients is adding other drugs to the conventional ones. The aim of this clinical trial study was to examine the effect of melatonin on in vitro fertilization (IVF) in PCOS women and evaluate the quality of the oocytes, embryo, and also the pregnancy outcome.

## MATERIALS AND METHODS

### 1. Study design, registration, and participants

This study was registered in Iranian Registry of Clinical Trials portal (IRCT20151123025202N11) and was approved by the Research Ethics Committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1399.302).

The sample size for testing the difference in means ( $\mu_1 - \mu_2$ ) between two populations (the level of estradiol in successful and unsuccessful pregnant groups) was calculated using the following formula. Mean and standard deviation indices were obtained from a similar study conducted by Jahromi et al.<sup>19</sup> for this test. The test power ( $Z_{1-\beta}$ ) was set

at 90%, and the level of significance ( $\alpha$ ) was set at 0.05.

$$n = \frac{(Z_{1-\alpha/2} + Z_{1-\beta})^2 \times [\delta_1^2 + \delta_2^2]}{(\mu_1 - \mu_2)^2}$$

All participants were informed before the start of the study that they would be randomly assigned to either the intervention group (pregnant women who would receive 3 mg of melatonin daily) or the placebo group (women who would receive metformin three times a day and 1 mg of folic acid daily). Then, after each individual's visit, they were assigned to one of the mentioned groups using one of the randomization methods (a combination of a table of random numbers and the block randomization method). The block randomization method is one of the best randomization techniques in intervention studies, which creates balance in the two groups with approximately equal numbers by considering a block size that is a multiple of the even number of treatments to maintain balance within the block. Blocks were selected using a table of random numbers, and finally, the number of patients was determined, and treatment types were placed on cards. The block size and number of blocks will be determined by the masker and were not known to the participants.

In this clinical trial, a total of 320 PCOS women, with aged 18-40 years were recruited between 2019-2020 at Endometrium and Endometriosis Research Center of Hamadan University of Medical Sciences. The diagnosis of PCOS was based on the Rotterdam criteria. It proposes a PCOS diagnosis when two of the three following factors are present in the patient: (a) oligo-anovulation, (b) biochemical or clinical hyperandrogenism, or (c) polycystic ovary morphology.

Because melatonin improves the effects of metformin in metabolic patients, women with PCOS were randomly assigned to the intervention and control groups. Patients in the intervention group (n=160) received a combination of melatonin and metformin as an insulin sensitizer (3 mg and 500 mg, respectively) three times a day. The control group (n=160) received metformin 500 mg from the luteal phase of the cycle before the start of gonadotropin. All patients provided written informed consent after counseling. All procedures performed in this study were in accordance with the 1964 Helsinki declaration and with the ethical standards of the National Research Committee. Patients were excluded if they had male infertility, tubal infertility, endocrine diseases, a history of hormonal drugs consumption in the last three months or drug interactions.

### 2. Treatment

Ovarian motivation was operated using the GnRH agonist long protocol or GnRH antagonist protocol; oocyte retrieval was performed 36 hours after HCG injection by transvaginal ultrasound. Then the oocytes were examined for maturity and grading and mature oocytes were injected (ICSI). Embryos were cultured in vitro after ICSI for three to five days. The embryos were graded, as follows: grade A:

**TABLE 1.** Demographic characteristics, infertility status of the intervention and control groups

	Group A (n=160) mean±SD/n (%)	Group B (n=160) mean±SD/n (%)	p-value
Patient age (years)	30.2±4.2	29.7±5	NS (0.318)
Age of husband (years)	34.9±5.1	34.8±4.6	NS (0.273)
Duration of infertility (years)	6.6±3.2	6.2±3.5	NS (0.843)
The number of oocytes in each patient	15.0±10.5	14.2±9.1	NS (0.46)
Total number of oocytes	2,650	2,150	0.001
Metaphase II	1,852 (69.9%)	1,244 (57.9%)	
Metaphase I	798 (30.1%)	906 (42.1%)	
Total number of embryo	1,440	1,120	0.001
Grade A	460 (40.3%)	335 (29.9%)	0.001
Grade B	680 (60.7%)	758 (70.9%)	
Clinical pregnancies, n	41 (25.6%)	26 (16.2%)	
Spontaneous abortion, n (%)	4 (2.5%)	6 (3.7%)	0.52

Data are presented as mean±SD. NS: no statistically significant. Group A: with Melatonin, Group B: without Melatonin.

Equally-sized blastomeres, round with no fragmentation, grade B: Slightly different blastomeres in size up to 10% with granules and vacuoles, grade C: Unequally-sized blastomeres up to 30% fragmentation with granules, and grade D: Unequally-sized blastomeres, extreme fragmentation dark and large granules and vacuoles.

Embryo quality was assessed before the transfer approximately 72 h (8-cell stage) after the insemination with a maximum of three embryos. Serum  $\beta$ -HCG levels were detected to determine the rate of biochemical pregnancy fourteen days after transplantation. Clinical pregnancy was set on by observing the fetus with cardiac activity at 6-7 weeks of pregnancy. Miscarriage was classified as pregnancy loss between the 5th and 12th week of pregnancy. Finally, both groups of patients were compared in terms of outcomes such as numbers and quality of oocytes and embryos, clinical pregnancies, and spontaneous abortion.

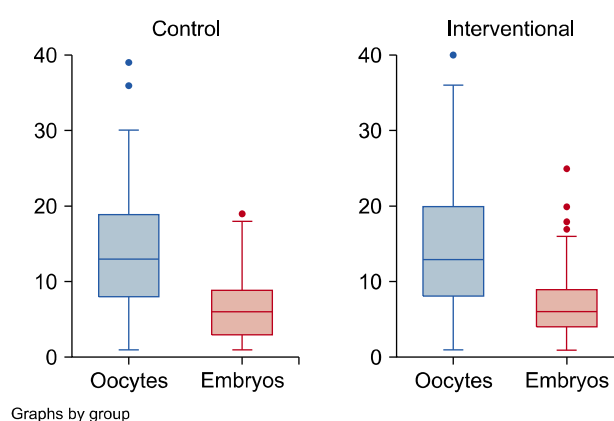
### 3. Statistical analysis

Descriptive statistics were reported as numbers (%) and means±SD for categorical and continuous variables. An independent t-test (t-test) was used to compare the quantitative variables at a significance level of 5%. All statistical tests were performed at the 95% confidence level using Stata software version 14.

## RESULTS

According to Table 1, both study groups were comparable in terms of mean age, duration and cause of infertility, age of spouse, number of oocytes and their maturity, embryo grading, clinical pregnancies, and spontaneous abortion.

The mean number of total oocytes retrieved did not differ between the two groups (14.2±9.1 vs. 15.0±10.5,  $p=0.46$ ) (Fig. 1). Whereas the group treated with melatonin reported a significantly greater mean number of mature oocytes (MII oocytes: 1,852 vs. 1,244,  $p=0.001$ ) and a lower mean number of immature oocytes (MI oocytes: 798 vs. 906,



**FIG. 1.** Box plot of the number of oocytes and embryos in patients of the intervention and control groups.

$p=0.001$ ).

In this study, 2,560 suitable oocytes and 1,440 embryos were generated, which belonged to the intervention group and 1,120 embryos belonged to the control group. Embryos classified due to their quality and the mean number of embryos of top-quality (grade A) was higher in the group treated with melatonin compared to the control (460 (40.3%) vs. 335 (29.9%),  $p=0.001$ ).

A total of 67 pregnancies were obtained (41 in group A and 26 in group B,  $p=0.039$ ). The rate of clinical pregnancy and implantation were higher in the group cotreated with melatonin ( $p=0.25$  and  $p=0.56$  respectively); although these differences did not reach statistical significance.

According to Table 2, using univariate logistic regression analysis, the odds of clinical pregnancy in the intervention group were 1.8 times of the control group, which was statistically significant ( $p=0.039$ ). The odds of spontaneous abortion were lower in the intervention group, but it did not show a statistically significant difference with the control group ( $p=0.520$ ).

**TABLE 2.** Pregnancy outcomes and spontaneous abortion of patients of the intervention and control groups

Pregnancy outcome	Group A (n=160)	Group B (n=160)	p-value	OR (95% CI)
Clinical pregnancies	(25.6%) n=41	(16.2%) n=26	0.039	1.8 (3.07-1.02)
Spontaneous abortion	(3.7%) n=6	(2.5%) n=4	0.52	1.5 (5.5-0.42)

Group A: with melatonin, Group B: without melatonin.

## DISCUSSION

Melatonin is a multifunctional molecule with various roles, including antioxidation, scavenging free radicals, regulating circadian rhythms, and as an anti-inflammatory. It is produced in the ovarian cells and affects the regulation of the reproductive system.<sup>20</sup>

Our study focuses on the protective effects of exogenous melatonin. The data revealed that melatonin treatment increased the number of mature oocytes compared to the control group (MII; 69.9% vs. 57.9%) and reduced the number of immature oocytes (MI; 30.1% vs. 42.1%). Our findings are consistent with other studies that reported almost the same results.<sup>21-24</sup> Melatonin can improve the number of top-quality embryos (grade A [40.3% vs. 29.9%]), clinical pregnancies (25.6% vs. 16.2%), and also reduce spontaneous abortions (2.5% vs. 3.7%). Our findings are consistent with other studies; they reported almost the same results.<sup>23,25</sup> In another study, the beneficial effects of melatonin on IVF patients were investigated. They concluded that the transfer ratio of grade A embryos was also significantly higher in the group treated with melatonin compared to the control group (69.3 vs. 44.8, respectively). Melatonin significantly facilitates the nuclear maturation of fresh human GV oocytes and early embryo by upregulating the clathrin-mediated endocytosis (CME), which improve maturation of oocytes by reducing the cAMP level and helping to reduce plasma membrane rigidity.<sup>26</sup> In other studies that used melatonin in an IVM culture medium; it was observed that melatonin reduces the oxidative stress and levels of early apoptosis, improves the mitochondrial integrity and spindle assembly, and aligns the chromosomes in oocytes. Also, it significantly increases the embryo growth in the laboratory condition.<sup>6,19,27,28</sup>

In PCOS patients, an increase in GnRH leads to an increase in LH and also change the ratio of LH/FSH; subsequently, androgen production by the theca cells increases in the ovaries, while FSH decreases and follicular maturation is significantly impaired. The action of both FSH and LH is required for follicular growth. Melatonin regulates GnRH through its MT1 and MT2 receptors leading to the inhibition of GnRH receptor expression and increased FSH. Finally, it leads to follicle growth and maturation, which includes follicle recruitment, selection, and ovulation.<sup>20,29</sup> In our study, melatonin significantly increased the number of mature oocytes.

The inhibition of GnRH receptor maintains the corpus luteum and increases the production of progesterone, which is an important factor in embryo implantation. Also,

due to the high amount of fibroblasts in the uterine horns of PCOS individuals, the collagen content is high and hinders the interaction of blastocysts in the uterus; it decreases the rate of implantation in these patients. Treatment with melatonin, especially in combination with metformin can reduce the high collagen fiber content of uterine horns and increases the rate of implantation.<sup>29</sup> Also, in our study, a higher rate of clinical pregnancy and implantation was observed in the group co-treated with melatonin.

Hyperandrogenemia in PCOS patients may be a predictor of obesity and Insulin Resistance<sup>30</sup> and appears that insulin-sensitizing agents play an important role in the treatment of these patients. IR leads to overexpression of vascular endothelial growth factor (VEGF), which is associated with infertility and a deficiency in oocytes quality. Melatonin affects the ovaries' follicular growth and steroidogenic activity by improving IR and glucose hemostasis and insulin secretion, reducing effects on hepatic gluconeogenesis, anti-hyperglycemic effects, and improvement of endothelial vascular function.<sup>31</sup>

ROSs are mediators of inflammatory responses and play an important function in ovulation. Neutrophils, vascular endothelial cells and macrophages, are located in the follicles and they can produce ROS.<sup>32,33</sup> Low levels of ROS play a role in the rupture of the follicle during ovulation, but high levels can lead to oxidative stress, which oxidize RNA, DNA, and proteins and damages the integrity of cell membranes oocytes and affects the granulosa cells and prevents the preparation of progesterone. Oxidative stress in the oocytes leads to failures in the creation of suitable embryos, because DNA damage and lipid peroxidation of membranes, which lead to inappropriate cell division and creation of fragmented embryos.<sup>34,35</sup>

Considering the increase in oxidative stress and decrease in antioxidant capacity in PCOS patients, as well as the decrease in melatonin concentration in the follicular fluid, melatonin administration is particularly important. It quenches ROS and RNS and protects GC and oocytes during ovulation. ROS leads to activation of the apoptosis cell signaling and peroxidative damages to the oocyte; it is involved in embryo fragmentation, arrest, or death.<sup>36</sup> Melatonin appears to protect follicles from oxidative stress and rescues their atresia. It also regulates gene expression of antioxidant enzymes (Catalase, SOD, GSH, and GPX).<sup>37</sup> In our study, mean number of top-quality embryos (grade A: equally-sized blastomeres, round with no fragmentation) was higher in the group treated with melatonin compared to the control.

Also, high levels of ROS dysregulate Ca<sup>2+</sup> homeostasis,

so it leads to more calcium loading and decreases mitochondrial membrane potential (MMP) in human oocytes. Mitochondrial damage leads to the release of cytochrome c and activation of caspases, and subsequently apoptosis,<sup>38</sup> but melatonin prevents apoptosis by increasing Bcl2 and reducing BAX and Caspase 3 by activating the PI3K/Akt pathway<sup>20,23</sup> and it impresses the oocytes mitochondria and reduces the excessive Ca<sup>2+</sup> levels and improves the maintenance of mitochondrial membrane potential.<sup>35</sup>

In this study, the total numbers of registered pregnancies were higher in patients treated with melatonin and indicated a positive effect of melatonin on the oocytes and the quality and outcome of pregnancy. Treatment with melatonin, especially with metformin, can reduce the high collagen fiber content of uterine horns and increase the rate of implantation.<sup>29</sup>

A comparison of the benefits of melatonin supplementation with other treatments that are usually used to improve the success rate of IVF in PCOS patients, such as Myo-inositol, vitamin E, statins, etc., was performed, it was found that melatonin in combination with Myo-inositol and Folic acid or vitamin E or metformin has better effects on oocyte quality and embryo fertilization.<sup>39,40</sup>

Finally, our study demonstrated that treatment with melatonin in PCOS patients can increase mature oocytes (MII) and reduce the number of immature (GV, MI) and degenerated oocytes; it can also improve the human oocyte maturation, fertilization, number of embryos of top-quality (grade A), clinical pregnancies, and reduce spontaneous abortions.

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## CONFLICT OF INTEREST STATEMENT

None declared.

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