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Research Article

Can Oral Melatonin Supplementation Affect IVF/ICSI Outcomes Through Changing Follicular Fluid Cytokines Level in Infertile Women? A Non-Randomized Controlled Trial

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Abstract

Melatonin is a natural hormone, which is synthesized primarily in the pineal gland and is derived from serotonin. In addition to antioxidant properties, much attention has been directed to the important immunomodulatory effects of melatonin. The aim of this study was to investigate the effects of oral melatonin supplementation on follicular fluid cytokines level and IVF/ICSI (*in vitro* fertilization/intracytoplasmic sperm injection) outcomes in infertile women. Thirty-eight infertile couples, who underwent IVF between May 2015 and February 2016 at the Infertility Center of Tehran Yas Hospital, were recruited for the study. The patients were divided into two groups: 17 women received oral melatonin treatment (3 mg/day for at least 3 weeks) while 21 women in the control group did not. We investigated the effects of oral melatonin supplementation on Interleukins (ILs) -10, -17, -21, -22 and Transforming Growth Factor Beta (TGF-β) concentrations in Follicular Fluid (FF) and ICSI outcomes of women undergoing IVF/ICSI cycles. None of the IL concentrations in follicular fluid was significantly affected by melatonin supplementation. We found that oral melatonin supplementation is not significantly associated with improving oocyte and embryo quality, fertilization, and biochemical pregnancy rates. Based on the present study, anti-inflammatory properties of melatonin may have no efficient effects on ICSI outcomes and likely, antioxidant properties of melatonin and decreasing oxidative stress may be involved, so additional studies in larger sample size are required for exploring effects of melatonin on IVF/ICSI cycles.

Keywords: IVF/ICSI; Melatonin supplementation; Interleukins; Follicular fluid; Infertility

Introduction

The quality of oocyte and embryo is an important factor affecting the pregnancy rate for couples undergoing infertility treatment. The development of infertility treatments and ART outcomes needs an appropriate strategy to improve this important factor. Many studies have evaluated the effects of supplementation therapy in order to increase the oocyte and embryo quality [1,2]. Protecting oocytes from oxidative stress is critical to access a successful fertilization, and a normal development of embryo and embryo transfer procedures [3].

Melatonin (N-acetyl-5-methoxytryptamine), as an indole amine is produced at pineal secretory, bone marrow cells, bile, and several reproductive organ sites such as oocyte, follicular cells of the ovary, preovulatory Follicular Fluid (FF) and placental cytotrophoblasts [3-7].

Melatonin is going to be an expected medicine of choice to improve the oocyte quality for women who cannot get pregnant because of poor oocyte quality [6,8]. The fertilization rate, pregnancy rate [8], and oocyte and embryo quality [9] have been improved with melatonin supplementation in patients with a lower fertilization rate during previous IVF/ICSI cycles [10]. Oral melatonin supplementation increases the level of intra-follicular melatonin followed by a reduction of intra-follicular oxidative damage [6].

Melatonin, functioning as a radical hunter is able to powerfully restrict free radical generation in order to protect oocytes from oxidative damage [3,11]. The presence of melatonin in culture medium and clinical implications suggests its improving role in oocyte maturation, and embryo development with a greater number reaching to the blastocyst stage, as well it decreases the mitochondrial activity [13] and oxidative damage to gametes happening at ovulation time [1,3,12].

The antioxidant capacity of melatonin is related to the neutralization of toxic oxygen-based reactants, the activity of free radical scavenging, the increase in the efficiency of electron shuttling, and the stimulation of glutathione synthesis [14-18].

In addition to antioxidant properties, much attention has been directed to the important immunomodulatory effects of melatonin [5]. T lymphocytes can be stimulated by melatonin through having membrane and nuclear receptors in order to facilitate the production

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Table 1: Demographic, hormonal data, and the causes and types of infertility of the patients who received melatonin (Intervention Group) and not received melatonin (Control Group).

Variable	Control group (21)	Intervention group (17)	
	Mean (±SD) (min-max)	Mean (±SD) (min-max)	P value Anova
Age (yr)	33.71 (±6.4) (22-44)	33.59 (±5.9) (23-42)	0.951
Duration of infertility (yr)	5.44 (±4.3) (1-15)	5.96 (±2.9) (1-11)	0.702
Serum LH level[1]	6.2 (±3.5) (.1-14.4)	4.4(±2.1) (1.9-8.9)	0.064
Serum FSH level[2]	9.3 (±4.9) (3.9-24.7)	7.6 (±3.0) (3.6-13.5)	0.221
Serum TSH level[3]	2.7 (±1.8) (.5-4.9)	2.4(±1.2) (0-7.1)	0.527
Serum PRL level[4]	84.5 (±153.1) (1.7-615)	56.9(±124.8) (4.1-466)	0.553
Cause of infertility	Control Group N (%)	Intervention Group N (%)	P value Chi-Square Test
Male	9 (42.9)	7 (41.2)	0.737
Female	7 (33.3)	7 (41.2)	
PGD	4 (19)	2 (11.8)	
Unexplained	1 (4.8)	1 (5.9)	
Total	21(100)	17(100)	
Type of infertility			
Primary	7 (35)	10 (58.8)	0.306
Secondary	9 (45)	4 (23.5)	
donor	4 (20)	3 (17.6)	
Total	20 (100)	17(100)	

[1]LH: Luteinizing Hormone (IU/L); [2]FSH: Follicle-Stimulating Hormone (IU/L); [3]TSH: Thyroid Stimulating Hormone (Miu/L); [4]PRL: Prolactin

of T helper 1 (Th1) cytokines [19]. In addition to stimulation of Th1 cytokines, melatonin has been reported to enhance T helper 2 (Th2) response in some circumstances [20]. Melatonin has also been reported to increase the production of IL-1, IL-6, and IL-12 in human monocyte cells as well as opioid cytokines in hematopoietic system [19,21,22]. With respect to immunomodulation, melatonin can suppress the production of pro-inflammatory cytokines such as tumor necrosis factor α, IL-1β, and IL-6 in some conditions such as liver injury [23-26]. As a consequence of melatonin action as an immune stimulant agent under basal or immunosuppressed conditions along with its anti-inflammatory effect in the presence of a transient or chronic exacerbated immune response, Carrillo Vico et al. used the term of "immunological buffering" in describing the melatonin activities [27].

Various factors can cause infertility in women, including immune conditions with a possibility of immunologic infertility [28]. According to antioxidant and immunomodulatory properties of melatonin, we investigated the effects of oral melatonin supplementation on oocyte and embryo quality, fertilization rate, and biochemical pregnancy with gonadotropin-releasing hormone agonist (GnRH-a) protocol in women undergoing ICSI cycle. As a mechanism for melatonin effects, we measured IL-10, IL-17, IL-21, IL-22 and TGF-β concentrations in FF of infertile women undergoing IVF/ICSI cycle and explored the association between melatonin supplementation and these FF Interleukins (ILs) concentrations.

Materials and Methods

Trial design

This study included 38 infertile women (aged 18-40 years) undergoing IVF/ICSI program at Infertility center, Tehran Yas Hospital and was approved by Ethic Committee of Tehran University of Medical Sciences (Trial registration: Current Controlled Trials: IRCT2015042912307N4). Prior to participation, informed consents were obtained from all patients. We enrolled women with following infertility criteria: male factor, tubal factor, and unexplained. Exclusion criteria were women with Polycystic Ovary Syndrome (PCOS), endometriosis, ovarian tumors, uterine malformation, genetic and coagulant disease, and also smokers. The patients underwent Controlled Ovarian Stimulation (COS). A single-blind non-randomized clinical trial was conducted. The patients, embryologist, and outcome assessor were blinded to assignment. By physician's decision, the women were included in two groups: 21 women were assigned to the intervention group receiving oral melatonin (Nature Made, USA) treatment (3 mg/day for at least 3 weeks) until the day of controlled ovarian stimulation with the injection of human Chorionic Gonadotropin (hCG). 17 women were assigned to the control group with only receiving a standardized ovarian-stimulation protocol.

Outcomes

Primary and secondary outcomes of this study were the number of mature oocytes at the Metaphase II Stage (MII), the quality of embryo, the serum estradiol and progesterone levels on the day of hCG administration, biochemical pregnancy rate and also, the comparison of IL-10, IL-17, IL-21, IL-22, and TGF-β concentration in FF.

Ovarian stimulation protocol

All patients received a standard GnRH agonist long protocol,

Buserelin acetate 500 µg/day (Suprefact, Aventis, Germany) starting at day 21 of a spontaneous menstrual cycle until pituitary down-regulation time (serum E2 <50 pg/ml in the absence of follicular structures larger than 10 mm). Then, the dose of Buserelin was reduced to 250 µg/day until the day of hCG injection when pituitary down-regulation was achieved. Ovarian stimulation was started on the 3rd day of the current menstrual cycle by injection of rFSH Follitropin alfa (Gonal F, Serono, Italy) at a daily dose of 150 to 225 IU in each group. By developing at least 3 follicles with a mean diameter of 17 mm (evaluated by transvaginal sonography), 5000 IU/2/IM of hCG (Choriomon, IBSA Institut Biochimique S.A., Switzerland) was injected. In both groups, the serum levels of 17-beta-estradiol (17-β E2) and progesterone were measured and recorded on the day of hCG administration.

Oocytes staging, Embryo grading and Embryo transfer

Oocyte retrieval was performed 36 hours after hCG injection under transvaginal ultrasound guidance. Cumulus and corona radiate cells were immediately removed after retrieval by a short exposure to Hyase 10X (Vitrolife; IVF Science, Sweden) medium and gentle aspiration by Pasteur pipette, then mechanically cleaned from the remaining surrounding cumulus cells by aspiration using a denuding pipette (stripper) (Swemed Denudation Pipette). Next, the total number of oocytes and their maturation status and quality were assessed under the magnification of 400 X with selecting oocytes having first polar body presumably at the metaphase II stage (MII) for ICSI. Oocytes were injected by sperms using the standard ICSI procedure. The embryos were incubated in the Global total Medium (Lifeglobal, Europe) covered with sterile mineral oil at 37°C with 6% CO_2 and 5% O_2, and their quality was assessed approximately 72 hours after ICSI. Luteal phase support was started on the day of oocyte pick-up with vaginal administration of 400 mg progesterone (Cyclogest, Actavis, UK) twice daily for up to 2 weeks.

Fertilization was evaluated through using an inverted microscope with Hoffman modulation contrast at 400 X magnifications (TE 2000 U, Nikon Corp., Japan) for about 16-18 hours after ICSI. Oocyte fertilization was confirmed by observation of 2 pronuclei (2PN) in the injected oocytes [29].

Evaluation of cleavage stage embryos was determined according to the Society for Assisted Reproductive Technology (SART) grading system and evaluated embryos were classified as good, fair and poor embryos [30,31].

About 12 days after embryo transmission, biochemical pregnancy was determined by a positive quantitative serum β-hCG assay.

Follicular fluid collection and cytokine determination by Enzyme-linked Immunosorbent Assay (ELISA)

Follicular fluid was collected from oocytes retrieval during transvaginal follicular puncture and centrifuged at 600 g for 10 minutes in order to remove cellular debris. The supernatant was frozen at −80°C until the process of cytokines analysis.

IL-10, IL-17, IL-21, IL-22 and TGF-β levels were detected in follicular fluid using commercial capture ELISA kits (eBioscience, San Diego, CA, USA), according to the manufacturers' protocol. Briefly, ELISA plate was coated with 100 µL/well of capture antibody presence in coating buffer and the sealing plate was incubated overnight at 4°C. The wells were aspirated and washed 3 times with 300 µL/well of wash buffer. Then, wells were blocked with 200 µL of ELISA/ELISASPOT Diluent (1X). The standard stocks were serially prepared to generate 8 points for the standard curves. After addition of 100 µL/well of samples to the appropriate wells, 100 µL of ELISA/ELISASPOT Diluent (1X) was added to the blank well and the plate was incubated at Room Temperature (RT) for 2 hours. Then, diluted capture antibody was added to each well at a final volume of 100 µl. After incubation and washing, 100 µL of diluted Avidin-HRP was added and the plate was again incubated at RT for 30 minutes. Next, TMB Solution (100 µL) was added and the incubation time was terminated after 15 minutes at RT with adding 50 µl of stop solution per well. Finally, the Optical Density (OD) of samples was read at 450 nm with an ELISA reader (ELx 800, BIO-TEK, USA). Sensitivity of the kits was 2 pg/ml for IL-10, 4 pg/ml for IL-17, 8 pg/ml for IL-21, 8 pg/ml for IL-22, and 156.3 pg/ml for TGF-β. All samples, standards and controls were assayed in triplicate.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences software (SPSS, version 16). All variables were tested for normal distribution with Kolmogorov-Smirnov test, histogram, and P-P plots. Variables were compared with either independent samples t-test or Mann-Whitney U test depending on the normality of the data. All categorical variables were compared with Pearson chi-square and Fisher's exact tests. A p value <0.05 was considered as statistically significant. Continuous variables were presented as mean ± SD.

Results

Demographic details namely age, serum levels of LH, FSH, TSH, and PRL on day 3 of the menstrual cycle, and the types and causes of infertility in the intervention and control groups are provided in Table 1. No significant differences were found in the mean age, serum levels of desired hormones on day 3 of the menstrual cycle, duration of infertility, and types and cause of infertility between the intervention and control groups.

Table 2 shows the primary and secondary outcomes in the intervention and control groups. The primary outcomes demonstrated that there were no significant differences in the mean number of retrieved mature oocytes between two groups (17±9.2 *vs*. 21±8.0, P > 0.05). Although the mean number of poor quality embryos was lower in the intervention group compared to the control group (0.41±0.8 *vs*. 0.5±0.7, P >0.05), this difference was not statistically significant.

Evaluation of the secondary outcomes revealed no statistically significant differences in 17-β E2 and progesterone levels on the day of hCG administration between two groups. There were also no statistically significant differences between the intervention and control groups with respect to the biochemical pregnancy rate.

Cytokine levels in FF

We evaluated the levels of TGF-β, IL-10, IL-17, IL-21, and IL-22 in the follicular fluid of infertile women undergoing IVF/ ICSI in melatonin and control groups (Table 3). There was no significant difference in the level of TGF-β in melatonin group (6.4±4.3 pg/ml) compared to control group (5.6±2.3 pg/ml). Although the level of IL-10 in melatonin group (2139.9±1446.2 pg/ml) increased compared

Table 2: Primary and secondary outcomes of the patients who received melatonin (Intervention Group) and not received melatonin (Control Group).

Variable	Control Group -21	Intervention group -17	P value
	Mean (±SD) (min-max)	Mean (±SD) (min-max)	Anova
*17 β-estradiol (Pg/ml)	1687.1 (±723.7) (746-3122)	2139.9 (±1446.2) (160-5849)	0.229
*Progesterone (ng/ml)	.8 (±.3) (.41-1.8)	.9 (±.3) (.28-1.7)	0.563
Total retrieved oocytes No.	21 (±8.0) (0-15)	17(±9.2) (0-20)	.401
**MII oocytes No.	21(±8.0) (0-15)	17(±9.2) (0-20)	.268
***2PN No.	5.6(± 2.3) (0-9)	6.4(± 4.3) (0-13)	0.474
Total Embryo No	5.4 (±2.6) (0-19)	6.2(±4.3) (0-13)	0.489
• Good quality embryo No.	4.1 (±2.7) (0-9)	4.8 (±3.7) (0-11)	0.527
• Fair quality embryo No.	.90(±1.1) (0-3)	1.06(±1.4) (0-4)	0.709
• Poor quality embryo No.	.5(±.7) (0-2)	.41(±.8) (0-2)	.789
	N (%)	N (%)	P value
Biochemical pregnancy rate	3(14.3%)	3(17.6%)	.560

*Serum levels on hCG day; ** Mature oocyte (Meiosis II); ***Two pronucleus

Table 3: Cytokines concentration in follicular fluid of patients who received melatonin (Intervention Group) and not received melatonin (Control Group).

Variable	Control Group (21)	Intervention group (17)	P value
	Mean (±SD) (min-max)	Mean (±SD) (min-max)	Anova
*IL 10 (pg/ml)	1687.1 (±723.7) (746-3122)	2139.9 (±1446.2) (160-5849)	0.627
IL 17 (pg/ml)	.8 (±.3) (.41-1.8)	.9 (±.3) (.28-1.7)	0.471
IL 21 (pg/ml)	21 (±8.0) (0-15)	17(±9.2) (0-20)	. 597
IL 22 (pg/ml)	21(±8.0) (0-15)	17(±9.2) (0-20)	. 786
**TGF-B (pg/ml)	5.6(± 2.3) (0-9)	6.4(± 4.3) (0-13)	0.653

*Interleukins, **Transforming growth factor beta

to control group (1687.1±723.7 pg/ml), but no significant difference was observed. The levels of IL-17 and IL-22 were non-significantly decreased (0.9±0.3 and 17±9.2 pg/ml) compared to those presenting in FF of control group (0.8±0.3 and 21±8.0 pg/ml). In addition, the level of IL-21 was non-significantly increased in the intervention group (17±9.2 pg/ml) compared to control group (21±8.0 pg/ml).

Discussion

Embryo quality is a useful predictor of successful IVF cycles and according to recent studies, there is a strong relationship between embryo quality, implantation, and clinical pregnancy rates. On the other hand, it has been shown that a poor quality of embryos can result in higher spontaneous abortions, and lower clinical pregnancies and live birth rates in women undergoing assisted reproductive techniques [32-34]. Thus, improving embryo quality is one of the most important goals in the infertility clinics and in recent years, many efforts have been made to obtain the best quality of embryos.

Based on evidences from studies, melatonin and its metabolites can protect the granulosa cells and oocytes during ovulation *via* suppression of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) [6]. Some studies showed that melatonin significantly increased the number of class 1 embryos in IVF patients, although the fertilization rate did not changed [9,35]. Giovanni Vitale et al. reported that an increase in the concentration of melatonin treatment in FF can decrease the oxidative stress and significantly improve the quality of oocytes and embryos [36]. A recent study showed that oral melatonin supplementation can improve oocyte quality, embryo quality and scavenging free radicals during ovarian stimulation for aged women undergoing ART [37]. Also, melatonin was reported to improve the outcome of IVF and embryo transfer with the protection of oocyte from toxic oxygen species [3].

Ovulation process is similar to a local inflammatory response [38], in which the production of ROS can be responsible for impaired oocyte quality. Oxidative stress induced by ROS during the process of ovulation has adverse effects on oocytes and granulosa cells, therefore it must be decreased in order to provide embryos with good quality [39]. Melatonin as an anti-inflammatory and antioxidant molecule having beneficial effects on reproductive physiology, can be employed in antioxidant therapy against oxidative stress conditions [8,40].

Cytokines are secreted proteins released by various cell types and have specific effects on the interactions between cells and physiological conditions.

An et al. investigated the levels of different cytokines in unexplained infertile women compared to healthy subjects and reported a high level of cytokines such as IL-21 suggesting them as new diagnostic indicators for screeningimmunologic infertility [41]. IL-17 is expressed by a distinct type of T cell, T helper 17 and other lymphocytes and is known as an inflammatory mediator in host defense and inflammatory diseases [42]. IL-22, a member of the IL-10 family, has important functions in tissue regeneration and host defense at epithelial tissues. Dambaeva et al. demonstrated a late-luteal expression of IL-22 and its role in endometrial regeneration. Moreover, they reported that its expression can be associated with

immune deregulation during the mid-luteal phase [43]. Additionally, other study indicated that the expression of IL-22 was higher in lesions of women with endometriosis than that from healthy women [44]. In Sabbaghi's study, the level of IL-22 was higher in FF of patients with endometriosis, Polycystic Ovary Syndrome (PCOS), and tubal factor infertility [45]. Ozkan et al. observed an increase in the plasma level of IL-17 in infertile women with a negative impact on ICSI outcomes [46]. In our study, we observed no significant decrease in IL-22 and IL-17 concentrations in FF of infertile women who received melatonin. We also found no significant increase in IL-21 level in intervention group.

Furthermore, we did not observe a significant difference in the levels of IL-10 and TGF-β between the two studied groups in infertile women undergoing IVF or ICSI. Similar to this study, Cerkiene et al. showed that there was no significant alteration in the concentration of IL-10 in FF of infertile women in different age groups [47]. Likewise, they demonstrated that the range of IL-10 was 0.7–10.8 pg / mL in 108 samples of human FF similar to our study with a range of 0.1-8.3 ng/ml measured for IL-10 [47]. However, in another study by Geva et al. concentrations of IL-10 in FF was ranged from 78.7 to 104.7 pg/ml [48]. It seems likely that the mentioned differences of IL-10 concentration in our study compared to Cerkiene et al. [47] and Geva et al. [48] can be related to the different types of material and methods we applied. Moreover, it is reported that melatonin may regulate TGF-β activity and then improve the ovarian function and oocyte quality [6]. Other study showed the lower expression of GDF-9 and TGF-β1 in the FF of women over the age of 35 underwent IVF which may support the role of TGF-β1 in reflecting the ovarian function [49].

The present study reported no significant differences in oocyte quality, pregnancy rates, the plasma concentrations of FSH, LH, TSH, PRL, 17 β-estradiol and progesterone, and Follicular Fluid (FF) levels of IL-10, IL-17, 1L-21, IL-22, and TGFβ after melatonin treatment between the intervention and control groups. However, our study found that the melatonin can non-significantly decrease the number of poor quality embryos in the intervention group compared to control group

Based on our findings, anti-inflammatory properties of melatonin may have no efficient effects on ICSI outcomes and so, antioxidant properties of melatonin and decreasing oxidative stress may be lead to a reduction in the number of poor quality embryos. However, more researches in large populations are required to confirm the effectiveness of melatonin supplementation on embryo quality. Hence, because of low sample size in our study and also for a better describing of melatonin benefits on ICSI outcomes, we strongly propose to repeat these evaluations in large scale studies.

According to our findings, there is no evidence supporting the fact that the quality of oocytes and embryos can be affected by anti-inflammatory properties of melatonin. Therefore, it is tempting to speculate that the anti-inflammatory effects of melatonin may have no important influence on the levels of ILs in FF of infertile women undergoing IVF or ICSI, indicating the possible effects of other pathways contributing in IVF outcomes.

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