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# Supplementation of IVF solutions with melatonin improves assisted reproductive technology results

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**Summary**

*Aim:* To evaluate the effects of melatonin supplementation of culture medium on the sperm survival rate, fertilization rate, cleavage rate, optimal embryo rate, blastocyst rate, implantation rate and clinical pregnancy rate. *Material and Methods:* Oocytes and sperm obtained from each patient (86 patients in total) were divided on average into four groups with different concentrations of melatonin (0 nmol/L, 0.1 nmol/L, 1.0 nmol/L, and 10.0 nmol/L). Melatonin was supplemented three times a day to maintain the concentration in different groups, and all the results were recorded at each related time. *Results:* The results indicated that melatonin could increase sperm survival rate, fertilization rate, and cleavage rate, and the solutions with 10 nmol/L melatonin were most effective ($p < 0.05$). Lower concentration of melatonin (0.1 nmol/L) might have negative effect on the fertilization rate. Melatonin could improve significantly optimal embryo rate, blastocyst rate, implantation rate, and clinical pregnancy rate ($p < 0.05$) and the peak values all appeared in the treatment group with 1.0 nmol/L melatonin. *Conclusions:* Addition of 1.0 nmol/L melatonin into culture media was the most effective to improve embryo quality and clinical outcome of IVF.

Key words: Melatonin; IVF; Fertilization rate; Embryo; Clinical pregnancy rate;

## Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is mainly secreted by pineal gland and has multifunctional bioactivities, including anti-oxidative, anti-inflammatory, anti-apoptotic, endocrinologic, and sleep-wake cycle [1, 2]. As an anti-oxidative, melatonin has its most unique characteristics compared with other oxygen scavengers, such as melatonin, is a direct free radical scavenger and removes reactive oxygen species through the special receptors [3, 4]. Additionally, melatonin also is a suicidal terminal scavenger of ROS, because it does not make itself become oxidative in the process of scavenging free radicals [5, 6]. Melatonin can also increase the activity of antioxidant enzymes including glutathione peroxidase and superoxide dismutase [7, 8]. Some scientists found that the concentration of melatonin in human preovulatory follicular fluid was higher than serum levels, and the levels of melatonin in ovarian follicles were increasing with the growth of follicles [9-11]. This may indicate that melatonin has an important role in the ovary. Researchers investigated the follicular fluid from 138 patients fertilized by intracytoplasmic sperm injection (ICSI). The results showed that the success of IVF was related to a significantly higher total antioxidant capacity and significantly lower level of ROS [12, 13].

Some scientists have investigated the effects of melatonin on in vitro fertilization and embryo transfer. Tamura *et al.* used melatonin of 3 mg/day in 115 patients who failed to achieve pregnancy and the fertilization rates of less than or equal to 50% in the previous cycle. They found that the fertilization rates were significantly higher when compared with their previous cycles [14]. Batıoğlu *et al.* found that patients given melatonin pills of 3 mg/day had higher percentage of mature oocytes, higher proportion of good embryo, and higher clinical pregnancy rate [15]. Other researchers investigated the effect of oral melatonin on the patients with sleep disturbances undergoing IVF. They found the oocyte retrieval rate, metaphase II oocyte rate, and good embryo rate significant increased [2].

Ovarian stimulation protocols could change the in vivo follicular environment and altering endogenous levels of anti-oxidant. Additionally, oocytes were no longer protected by follicular fluid containing rich anti-oxidant and exposed in the environment with high level of oxygen, causing oocytes and embryos to be damaged by high level of ROS in the process of IVF [16, 17]. Oxidative stress affected the development and quality of oocytes and embryos, which decreased the fertilization rate and the success of IVF [14, 18, 19]. Therefore some investigators added melatonin into culture media to protect oocytes and embryos and improve the success rate of IVF. For example, researchers found that melatonin added into culture media with an optimal threshold of $10^{-5}$ to $10^{-9}$ M could improve MII oocyte rate, implantation rate, and clinical pregnancy

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rate, and higher concentration of melatonin would be harmful to the outcomes of IVF [20, 21]. Kim *et al.* also found that addition of melatonin into in vitro maturation (IVM) medium could improve the cytoplasmic maturation of human immature oocytes and subsequent clinical outcomes [20]. Wei *et al.* showed that low concentration of exogenous melatonin could improve nuclear maturation of human oocyte during rescue IVM, while high concentration of melatonin presented negative effects [21].

The plasma membrane of sperm contains a lot of polyunsaturated fatty acids which is important for fusion with the oocyte and fertilization, making sperm more easily be damaged by ROS. Researchers found that DNA fragmentation coming from both in vivo and in vitro oxidative stress was the major reason for poor sperm quality and function [22], and DNA damage had a adverse impact on fertilization, blastocyst development, miscarriage rates, and pregnancy outcome [23, 24]. Researchers found that human seminal fluid contained melatonin and spermatozoa express melatonin receptors [25, 26]. Addition of melatonin to seminal samples could increase the percentage of sperm motility, reduce the proportion of non-viable sperm, protect sperm from oxidative damage, and enhance sperm quality [27-29]. Melatonin also appeared to reduce the early apoptotic events and prolong sperm survival [27, 30]. These authors thought the reason might be that melatonin could neutralize reactive nitrogen species and reduce the damage of reactive nitrogen and oxygen species to sperm to increase sperm quality.

The outcomes of IVF are related to the levels of ROS which have negative impact on the quality of sperm, oocytes, and embryos. The unique antioxidative characteristics of melatonin make it possible to be used in the process of IVF to reduce oxidative damage. However, there has been less research investigating the effects of addition of melatonin into IVF media on human oocyte and embryo, and subsequent clinical outcome. The purpose of this study was to investigate the effects of addition of melatonin to IVF media on the process of IVF-ET and confirm the optimal effective dose.

## Materials and Methods

In this study, the authors enrolled 86 infertile couples undergoing IVF cycles at the center of reproductive medicine, according to the following criteria: 1) age from 30 to 35 years, 2) patients undergoing their first IVF cycles, 3) only tubal factor, and 4) fresh embryo transfer cycle and transferring two embryos at the third day. All patients gave a written informed consent to participate in this study, and the study design was approved by the local institutional ethics committee.

The patients were treated with gonadotropin-releasing hormone analogue triptorelin acetate from either the preceding mid-luteal phase in a long treatment protocol. Ovarian stimulation was carried out with recombinant human FSH. Follicular development was monitored with serial vaginal ultrasound examinations and serum estradiol measurements. Human chorionic gonadotropin (hCG, 10,000 IU) was administered intramuscularly when the dominant follicles reached 18 mm in diameter and at least circum two follicles were > 17 mm in diameter. Oocytes were retrieved with transvaginal ultrasonographic guidance 35 hours after injection of hCG. The luteal support was initiated on day 1 after oocyte retrieval with 60 mg/day of progesterone, which was administered until the measurement of serum beta-hCG on day 14 and prolonged until 12 weeks in cases of pregnancy.

Sperm and oocytes obtained from 86 infertile couples were divided on average into four groups with different concentrations of melatonin (0 nmol/L, 0.1 nmol/L, 1.0 nmol/L, and 10.0 nmol/L), respectively. Human sperm survival testing, IVF, and embryo culture were executed according to the standard procedure of the center, except adding melatonin in the culture media. Human sperm survival testing was executed at room temperature, and oocytes and embryos were cultured in an atmosphere of 5% $O_2$, 6% $CO_2$, and about 95% relative humidity at 37°C. Sperm survival rate, fertilization rate, cleavage rate, and good embryo rate were evaluated at 72, 16-18, 43-45, and 67-69 hours, respectively. Blastocyst rate were evaluated at the fifth or sixth day.

Statistical analysis was carried out using the Statistical Package for the Social Sciences software (SPSS, version19.0). Data are shown as mean ± SD. Variables were compared with independent samples *t*-test and categorical variables were compared with Pearson chi-square and Fisher's exact tests. A $p$ value less than 0.05 was considered statistically significant.

## Results

Compared to the control group (0 nmol/L), sperm survival rates were incremented with the increasing of melatonin concentration, and the difference all achieved a significant level ($p < 0.05$) (Figure 1A). The results indicate that addition of melatonin into sperm culture media could enhance sperm quality.

Compared to the control group, melatonin had a significant effect on the normal fertilization rate of human ($p < 0.05$), and the normal fertilization rate increased significantly (0 nmol/L melatonin) in the group with 10 nmol/L melatonin (Figure 1B,). Comparing with the treatment group with 1.0 nmol/L melatonin, the normal fertilization rate decreased in the treatment group with 0.1 nmol/L melatonin, but the difference did not achieve a significant level ($p > 0.05$). Figure 2 shows that the cleavage rate of the treatment group with 10 nmol/L melatonin was higher compared to the control group ($p < 0.05$), and other treatment groups had no significant difference with the control group ($p > 0.05$). The results indicate that 10 nmol/L melatonin in media could significantly improve cleavage of human zygote.

Compared to the control group, melatonin had a positive effect on the optimal embryo rate and the difference reached a significant level in the treatment groups with 0.1 nmol/L melatonin ($p < 0.05$) (Figure 3A). Melatonin also improved the blastocyst rate and the differences achieved significant levels in the treatment groups with 0.1 nmol/L and 10 nmol/L of melatonin ($p < 0.05$) (Figure 3B). The results indicate that 1.0 nmol/L melatonin might be the optimal concentration to improve the formation of optimal

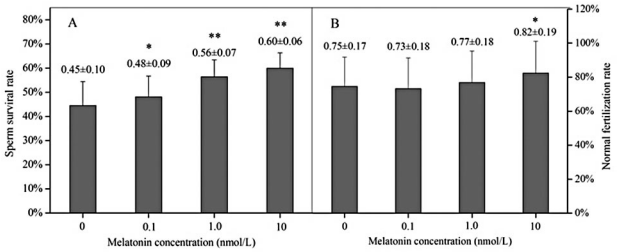


Figure 1. — Effects of different concentrations of melatonin on sperm survival rate in humans.
*$p < 0.05$ and **$p < 0.01$ compared to control.

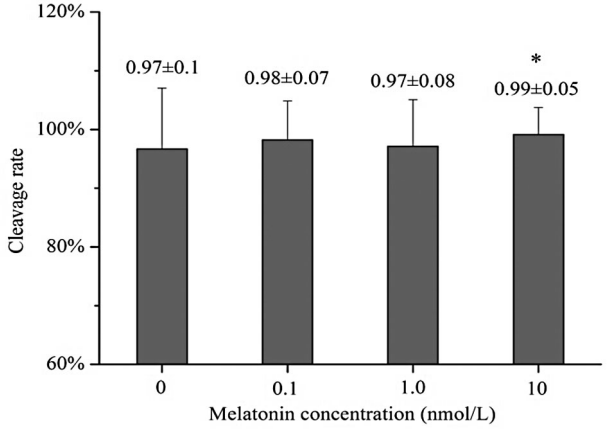


Figure 2. — Effects of different concentrations of melatonin on the cleavage rate in humans.
*$p < 0.05$ and **$p < 0.01$ compared to control.

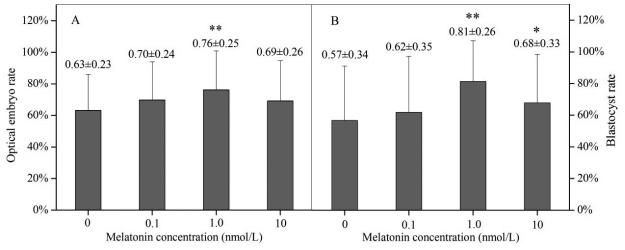


Figure 3. — Effects of different concentrations of melatonin on the optimal embryo rate and blastocyst rate of human.*p < 0.05 and **p < 0.01 compared to control.

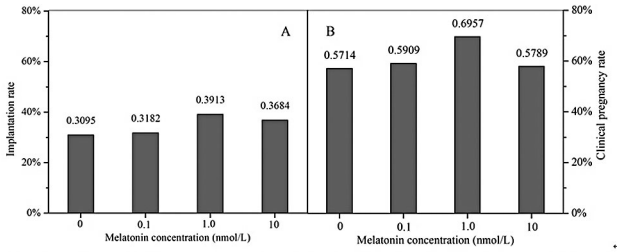


Figure 4. — Effects of different concentrations of melatonin on the implantation rate and clinical pregnancy rate in humans.

embryo and blastocyst. Compared to the control group without melatonin, the implantation rates and clinical pregnancy rates were all higher in the treatment group with different concentrations of melatonin (Figure 4). The implantation rates and clinical pregnancy rates were the highest in the treatment group with 1.0 nmol/L melatonin, but the differences were not significant at 0.05 level. The results indicate that optimal concentration of melatonin to improve the implantation rates and clinical pregnancy rates was about 1.0 nmol/L, and lower and higher melatonin were mostly ineffective. Concentration of 1.0 nmol/L could most effectively increase the optimal embryo rate and blastocyst rate (Figure 3), which indicates that embryos had a relatively high developmental potential and clinical pregnancy rate in the treatment group.

## Discussion

Melatonin had positive effects on embryos both in animal models and human assisted reproductive treatments, and this had been reported by many studies [14, 15, 20]. In the present study, similar results had been found, that melatonin benefited human embryos in assisted reproductive treatment.

The present study showed that the sperm survival rate (72 hours) of melatonin groups was higher than the non-melatonin group, and the differences were significant, especially, the difference between non-melatonin group and 10 nmol/L melatonin ($p < 0.01$, Figure 1A). From Figure 1B, the data suggests that 10 nmol/L of melatonin was related to a higher normal fertilization rate, and the cleavage rate in was also the optimal result in this group. The 1.0 nmol/L melatonin group had good performance in optimal embryo rate and blastocyst rate, the implantation rate, and clinical pregnancy rate reached the highest level, but the differences were not significant.

Due to a short half-life of melatonin, in this study the authors administered supplementation of melatonin three times a day, and frequent operations might affect embryos negatively.

Agarwal *et al.* showed that repeated cycles of centrifugation for separating sperm significantly increased ROS levels in human sperm and the presence of excessive ROS in semen had positive correlation with low sperm concentration, poor motility, and poor morphology [31]. Increasing the level of melatonin had positive effect on sperm quality and protected sperm from the oxidant environment produced by excessive round cells in seminal fluid [28]. Ortiz *et al.* also reported that both melatonin and antioxidant endogenous levels had positive correlation with seminal parameters, including sperm concentration, motility and morphology, and negative correlation with round cells [28]. Other research also showed that melatonin could increase the percentage of sperm motility and decrease the proportion of non-viable sperm [27-29], which was similar

to the present results. As a suicidal terminal scavenger of ROS, melatonin could reduce the damage of ROS to sperm and adjust sperm activity by its special receptors on human sperm [26, 28, 29]. These might be the major reasons that melatonin improved sperm quality. Therefore, melatonin is a powerful antioxidant and could improve sperm quality, which made it beneficial to obtain a successful ART outcome.

Previous research showed that low concentration of exogenous melatonin could improve nuclear maturation of human oocyte during rescue IVM, while higher concentration of melatonin presented negative effects [21, 32]. Studies on other animals also indicated that higher and lower doses in in-vitro maturation media had negative effects on the maturation rate of mice oocytes [33, 34]. The present study might indicate that lower concentration melatonin in culture media could not scavenge effectively the ROS and had negative effect on the maturation of human oocytes, so that the fertilization rate in the media with 0.1 nmol/L melatonin was lower than the other groups. Proper concentration of melatonin could decrease ROS production, improve sperm quality, and oocytes maturation rate, which might be the major reason of higher fertilization rates in the media with 1.0 and 10 nmol/L melatonin.

Studies in bovine and mice also showed that addition of melatonin increased cleavage rate and blastocyst formation rates [35].

Ali *et al.* ann Khalil *et al.* studies indicated that decreased antioxidant activity and increased ROS in oocytes were responsible for defective embryo development, apoptosis, and embryonic arrest [36, 37]. Melatonin could regulate the expression of pro-apoptotic genes (BAX and caspase-3) and anti-apoptotic gene (Bcl-2), and reduce the production of ROS. That would be beneficial to elevate cell number in blastocysts, improve embryo quality, and efficiency of embryo implantation [38-41]. Other research has also indicated that low concentrations ($< 10^{-9}$ M) melatonin might be ineffective to scavenge ROS, and higher concentrations ($> 10^{-9}$ M) melatonin might result in cell injury and lower the blastocyst rate. Study in mice indicated that melatonin could improve blastocyst rate, and the number of trophectoderm and inner cell mass showed a significant increase in $10^{-9}$ M melatonin [42]. Therefore, the optimal concentration of melatonin in both the activation and culture medium seems to about $10^{-9}$ M [43, 44]. These results all were similar to the present authors' studies.

Kim *et al.* showed that addition of melatonin into in-vitro culture medium could facilitate the cytoplasmic maturation of human immature oocytes and improve subsequent implantation rate and clinical pregnancy rate in IVF patients with polycystic ovary syndrome (PCOS) [20]. Other researchers showed that oral melatonin increased the intrafollicular concentrations of melatonin, reduced intrafollicular oxidative damage, and elevated fertilization and pregnancy rates [3, 15]. These results all indicated that supplementation with melatonin would be useful to improve the clinical outcomes of IVF.

## Conclusion

The present study indicated that addition of melatonin in culture media could improve sperm quality, fertilization rate, optimal embryo rate, blastocyst rate, sequent implantation rate, and clinical pregnancy rate. Optimal concentration of melatonin added to culture media to improve clinical outcome of IVF was 1.0 nmol/L, and lower and higher melatonin concentrations were not the most effective. Overall, only a limited number of clinical studies have investigated the effect of melatonin on improving clinical outcomes of IVF, and more clinical research is required.

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