**Original Article**

# Evaluating The Effect of Melatonin on *HAS2*, and *PGR* Expression, as Well as Cumulus Expansion, and Fertility Potential in Mice

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Abstract

**Objective:** Infertility is a worldwide health problem which affects approximately 15% of sexually active couples. One of the factors influencing the fertility is melatonin. Also, protection of oocytes and embryos from oxidative stress inducing chemicals in the culture medium is important. The aim of the present study was to investigate if melatonin could regulate hyaluronan synthase-2 (*HAS2*) and Progesterone receptor (*PGR*) expressions in the cumulus cells of mice oocytes and provide an *in vitro* fertilization (IVF) approach.

**Materials and Methods:** In this experimental study, for this purpose, 30 adult female mice and 15 adult male mice were used. The female mice were superovulated using 10 U of pregnant mare serum gonadotropin (PMSG) and 24 hours later, 10 U of human chorionic gonadotropin (hCG) were injected. Next, cumulus oocyte complexes (COCs) were collected from the oviducts of the female mice by using a matrix-flushing method. The cumulus cells were cultured with melatonin 10 µM for 6 hours and for real-time reverse transcription-polymerase chain reaction (RT-PCR) was used for evaluation of *HAS2* and *PGR* expression levels. The fertilization rate was evaluated through IVF. All the data were analyzed using a t test.

**Results:** The results of this study showed that *HAS2* and *PGR* expressions in the cumulus cells of the mice receiving melatonin increased in comparison to the control groups. Also, IVF results revealed an enhancement in fertilization rate in the experimental groups compared to the control groups.

**Conclusion:** To improve the oocyte quality and provide new approaches for infertility treatment, administration of melatonin as an antioxidant, showed promising results. Thus, it is concluded that fertility outcomes can be improved by melatonin it enhances *PGR*.

***Keywords:*** Hyaluronan Synthase-2, Melatonin, Mouse Oocyte, Progesterone Receptor

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## Introduction

As defined by world health organization (WHO) infertility is as the inability of a couple of child-bearing age to conceive over 12 months of regular unprotected sexual intercourse and is considered as a public health problem (1). About 10-15% of young couples have been reported to suffer from this conditions. Of these, 40-55, 20-30, and 15-17% are due to female factors, male factors and unexplained conditions, respectively (2, 3).

For 10% of couples trying to conceive underlying cause of the infertility are not easily identifiable, though all their relevant tests are normal. In this case, *in vitro* fertilization (IVF) may be recommended since it has shown to be the most effective treatment for unexplained infertility (4). Conditions such as exposure to light, elevated oxygen concentrations, and unusual concentrations of metabolites can cause an oxidative stress in oocytes and embryos during *in vitro* culture. Reactive oxygen species (ROS) can damage cell membranes and DNA and cause apoptosis (5). Therefore, protecting oocytes and embryos from oxidative stress in the culture medium is of crucial importance. In this regard, the usage of antioxidant compounds may result in more favorable results.

Oxidative stress can be reduced and culture conditions can be promoted an antioxidant or a radical scavenger is added to in the *in vitro* culture medium. As the main hormone secreted by pineal gland in the human brain, melatonin (N-acetyl-5-methoxytryptamine) is an endogenous compound which was discovered about 50 years ago. Through its stimulatory actions on the antioxidant system, it is regarded as a direct free-radical scavenger and an indirect antioxidant (6-8).

Several studies have demonstrated that melatonin is a powerful direct free-radical scavenger. In contrast to a majority of other known radical scavengers, melatonin is a universal multifunctional antioxidant. Melatonin has been reported to act as a hydrophilic and hydrophobic antioxidant since it is both lipid and water soluble (9). Also, the presence of melatonin receptors in the ovary at multiple sites, indicates that melatonin may influence the reproductive system (10). Melatonin concentrations in ovarian follicles increase with

follicular growth (11). Furthermore, melatonin treatment enhances the hCG-stimulated progesterone secretion (12). Earlier studies indicated that progesterone, a steroid hormone is a key player in ovulation. The biological effects of progesterone are mediated via *PGR*, a ligand-activated transcription factor. Ovarian *PGR* expression is undetectable during follicular development (13). It is likely that some *PGR*-regulated factors are secreted by granulosa cells and delivered to other cell types, such as cumulus cells within the ovary. Furthermore, besides playing a critical role in the ovary, progesterone is known to be essential for the maintenance of pregnancy. *PGR*s as members of the nuclear receptor superfamily of transcription factors (*NR3C3*) mediate progesterone effects (14).

*PGR* has been identified as an important regulator of gene transcription during the peri-ovulatory period, especially *PGR* regulates the transcription of the genes required for a successful oocyte release from the preovulatory follicle. In response to luteinizing hormone (LH), granulosa cells release epidermal growth factor (EGF)-like ligands, which in turn, cause cumulus cells to undergo an expansion (15). The cumulus oocyte complexes (COCs) formed due to the presence of a unique extracellular matrix plays an important role in successful oocyte maturation and ovulation (16). At the same time, the oocyte resumes meiosis and undergoes a maturation process to become competent for ovulation, fertilization, and expansion of COCs. During the expansion of cumulus cells, *HAS2* is the most important gene involved in the production of hyaluronic acid (HA) matrix (17).

It is generally accepted that *HAS2* mRNA is a key element required for the cumulus expansion process, which is necessary for oocyte maturation and ovulation (17-21). Increasing cumulus cell expansion can improve fertility rates because *HAS2* and *PGR* expression rates are enhanced during cumulus expansion. Melatonin may influence the cumulus expansion by augmenting *HAS2* and *PGR* expressions. Therefore, the effects of melatonin on *HAS2* and *PGR* expression and cumulus expansion were evaluated in this study.

## Materials and Methods

This experimental study was approved by the Research Committee of Tabriz Medical University according to the rules of the Ethics Committee. Forty five adult mice (15 male and 30 females, 30-35 g and 6-8 weeks old) were obtained from the animal house of Tabriz University of Medical Sciences (TUMS). Animals were kept in a 12-hour light/12-hour dark cycle with an unrestricted access to food and water at room temperature for 2 weeks.

Preparation of melatonin stock solution was done using an ethanol/TCM199 system. For this purpose 23.23 mg of melatonin (Sigma, United states) was dissolved in 1 ml of 0.1% absolute ethanol and diluted with TCM199 and serial dilutions were prepared. Using this method, a stock solution of melatonin 10 μM was prepared (22) and stored in a refrigerator at 4˚C at most for 2 weeks. In our experiment, ethanol amount was 0.1% in the maturation medium.

All the animals were treated in accordance with the guidelines of University Ethics Committee for care and use of laboratory animals. The female mice were superovulated using an intraperitoneal injection of 10 IU human menopausal gonadotropin (HMG) (NV Organon, Oss, The Netherlands) and after 24 hours, another intraperitoneal injection of 10 IU of human chorionic gonadotropin (hCG) (NV Organon, Oss, Holland) was done.

The female mice were sacrificed within 48 hours of hCG injection, and the ovaries and oviducts were removed after. Next, the mice ovaries (n=60) and oviducts were placed in sterile phosphate-buffered saline (PBS, Sigma, USA). Then, samples were transported to the Tissue Engineering Laboratory. Upon removing the stromal tissues surrounding the oviducts, the oocytes were collected from the uterine tube using the flushing method (universal medium of Azar Panam). By using a head sampler, the oocytes were drawn and poured into a dish. Finally liquid oil was added to prevent the culture medium evaporation.

The cumulus cells surrounding the oocytes, (i.e., cumulus oocyte complexes (COCs) were transferred to another dish containing the medium and then PBS was added for washing. In the experimental groups, the cumulus cells were cultured in the medium supplement with 10 μM melatonin for 6 hours. For evaluation of cumulus expansion, COCs were morphologically classified at recovery as having a compact or expanded investment. COCs were cultured in 200 mμ of a universal IVF medium and then incubated with sterile mineral oil at 37˚C with 5% $CO_2$ for 6 hours. After this incubation period, the medium was centrifuged twice at 3000 rpm for 5 minutes. Afterwards, the pellet of the cumulus cells was transferred to -20˚C for 1 hour and then stored at -80˚C until RNA isolation.

### *In vitro* fertilization

The animals selected for IVF were divided into control and experimental groups. After superovulation, the female mice were killed by cervical dislocation, their oocytes were collected by uterine tube flushing. Next, sperms were collected from the caudal epididymis of male mice by incubating the pieces of epididymis with Ham's F-10 medium culture using a $CO_2$ incubator at 37˚C for 20 minutes. The sperm samples were added to the collected oocytes of the control and experimental groups. The rate of fertility success was evaluated based on embryo formation associated with more cleavages and morula.

### Real time reverse transcription-polymerase chain reaction

To measure *HAS2* and *PGR* mRNA in the control and experimental groups, RNA expression was determined by using real time reverse transcription-polymerase chain reaction **(**RT-PCR) assay. The primers used in the PCR are presented in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the internal control gene to normalize the results. By using RNeasy Micro kit (CinnaGen, Iran), the total RNAs of *HAS2* and PRs were extracted. Primers were designed and real-time RT-PCR was conducted to analyze the gene expressions using SYBR green technology.

**Table 1:** Primer process for real-time reverse transcription-polymerase chain reaction

| Gene | Sequence (5´-3´) | Start | Stop | Product length |
|---|---|---|---|---|
| *HAS2* | F: CCTCACTGCGCAGACTACCA | 3876 | 3895 | 131 |
| | R: CCATACGGCGAGAGTCGGAG | 4006 | 3987 | |
| *PGR* | F: TGTTGTCAGGCTGGCATGGT | 2493 | 2512 | 182 |
| | R: AGTGGCGGGACCAGTTGAAT | 2674 | 2655 | |
| *GAPDH* | F: CGGGGTCCCAGCTTAGGTTC | 30 | 49 | 103 |
| | R: GCCCAATACGGCCAAATCCG | 132 | 113 | |

### Statistical analysis

Using SPSS software, version 22 and the sample t-test, all the statistical analyses were performed. P<0.05 were considered statistically significant.

## Result

### Effect of melatonin on *HAS2* and *PGR* expression in cumulus cells

*HAS2* and *PGR* mRNA expressions in cumulus cells isolated from mice oocytes were evaluated using quantitative real-time RT-PCR. A significant increase was observed in *PGR* mRNA in the experimental compared to the control group (Fig.1). However, no significant differences in *HAS2* gene expression were observed between the experimental and control groups (Fig.2). At this stage, each of the control and experimental groups were processed for real-time RT-PCR assay for 3 times. Hence, 3 experimental and 3 control groups are shown for each gene in the diagram (Figs.1, 2).

### Melatonin effect on fertilization rate and cumulus expansion

IVF was carried out in both control and experimental groups and 100 oocytes were evaluated from each group. By counting the number of embryos, it was revealed that more oocytes were developed into the embryos in the experimental group receiving melatonin for 6 hours as compared to the control group which did not receive melatonin (P<0.05). The embryos developed in the experimental and control groups are shown in Figures 3 and 4, respectively. Moreover, compact cumulus was seen to be tightly attached to the cells surrounding the smooth-surfaced oocytes over the cumulus hillock as shown by evaluating of the uniformity of ooplasm. On the other hand, expanded cumulus cells were detached from the oocytes with a matrix visible between the cumulus cells. The numbers of the expanded and compact COCs are demonstrated in Table 2. The statistical analysis showed that embryo formation in the experimental group was significantly increased (P<0.05, Table 2).

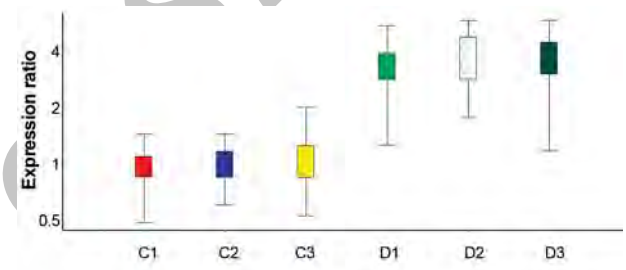


**Fig.1:** *PGR* gene expression in isolated cumulus cell in control groups (C) and in groups received melatonin (D).

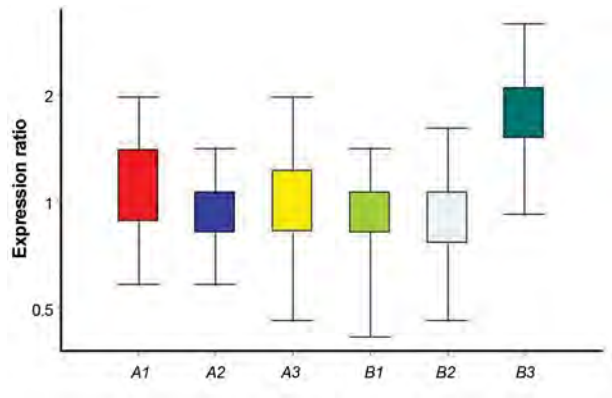


**Fig.2:** *HAS2* gene expression in isolated cumulus cell in control groups (A) and in groups received melatonin (B).

**Table 2:** Embryo formation percentage in the control and experimental groups

| Oocyte | Experimental Group (%) | Control Group (%) | P value |
|---|---|---|---|
| Fertilization | 80 | 77 | <0.05 |
| Oocyte expansion rate | 64 | 36 | <0.05 |

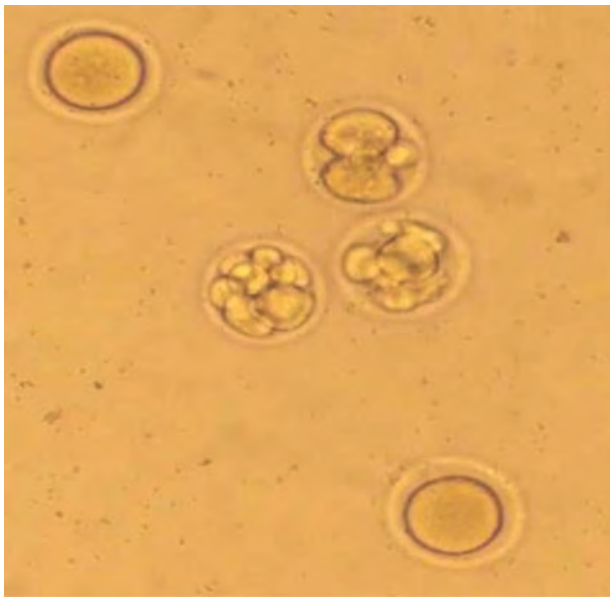

**Fig.3:** A photomicrograph of inseminated oocytes in melatonin-treated mice.

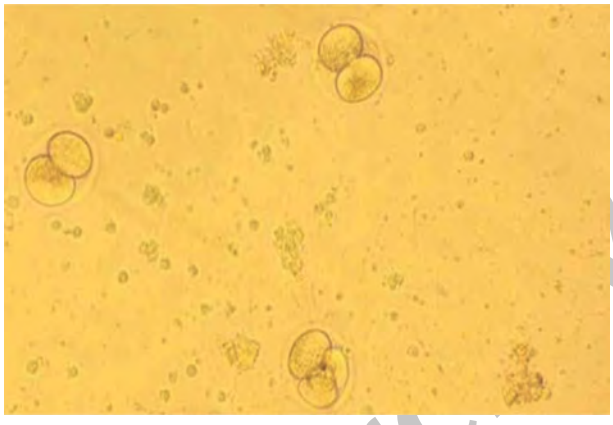

**Fig.4:** A photomicrograph of inseminated oocytes in control group.

## Discussion

It is generally accepted that melatonin and the presence of its receptors in granulosa cells can potentially exert beneficial effects on the ovarian function (22). As melatonin is a powerful free-radicals scavenger which is even more potent than vitamin E (23), it might be able to protect granulosa cells from the cytotoxicity of free-radicals that could be produced following long-term *in vitro* culture (24, 25).

The results of the present research are in agreement with previous histological and immunohistochemical studies, which showed that free-radicals scavenger could promote the qualities of the cumulus-oocyte complexes through inducing a uniform distribution of follicle cells covering the oocytes in the ovaries (26). These findings are in line with those reported the study of Bahadori et al. (9), describing the detrimental effect of oxidative stress on oocyte microenvironment and subsequently on implantation, follicular development, ovulation, oocyte quality, and early embryonic development. Also, the results of the study conducted by Ishizuka et al. (27) is consistent with those of the current research indicating that melatonin supports both mice fertilization and early development of embryonic tissue in the culture medium.

However, no relationships between oxidative stress markers and pregnancy rate were reported by Jozwick et al. (28) *HAS2* gene expression in cumulus cells is critical for the formation of hyaluronan, the predominant matrix component surrounding the expanded cumulus cells. Hyaluronan synthesis, which appears to occur at the transcription level of *HAS2*, is strictly regulated in cumulus cells. The present study confirmed the role of melatonin in promoting cumulus cell expression of *HAS2* though the influence was not significant (29-31). The results of another study showed that oocyte quality is ameliorated by antioxidants, while it directly increases the degrees of cumulus cell expansion and *HAS2* expression (32). Moreover, observed in our study, it was previously shown that in that melatonin increases the number of *PGR*s (33). The present study revealed the effects of administration of melatonin on IVF, cumulus cell expansion, maturation, and *HAS2* and *PGR* expressions.

## Conclusion

It is concluded that melatonin can improve IVF outcome by increasing cumulus cell expansion and *PGR* expression, while it had no influence on *HAS2* expression.

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## Author's Contributions

M.E.; Participated in executing the plot. L.R.; Contributed to the design and executive process. M.E., L.R.; Involved in writing and editing the manuscript and preparation of figures. J.S.R.; Participated in the design and conception. N.K.; Conducted in real time RT-PCR technique. All authors read and approved the final manuscript.

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