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Article

The effect of melatonin on *in vitro* maturation fertilization and early embryo development of mouse oocytes and expression of *HMGB1* gene in blastocysts

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Antioxidants are commonly used for maturation, fertilization and early development of embryos. Melatonin as an antioxidant have been recently proven to be useful for the assisted reproductive technology. In the present study, we evaluated the roles of melatonin in the *in vitro* maturation, fertilization, development and also the gene expression of high mobility group box-1 (*HMGB1)* in the blastocysts. The immature oocytes of BDF1 mice were transferred to the media containing different doses of melatonin (10^{-6}, 10^{-9}, 10^{-12} M). The blastocysts that developed under *in vitro* fertilization from each group were stained to determine the cell number of embryos and analyzed to determine the expression level of *HMGB1* by real-time PCR. The most effective doses of melatonin for maturation of oocytes were 10^{-6} and 10^{-12}M ($P<0.05$). Fertilization rate, early development and the cell number of blastocysts were significantly higher in the group that treated with 10^{-12} M of melatonin comparing to the other groups. The *HMGB1* expression decreased in groups that treated with 10^{-6}M and 10^{-9}M of melatonin and increased in the group that treated with 10^{-12} M of melatonin, but did not show a significant difference ($p>0.05$). From the results, it may be concluded that the melatonin could be effective when the embryos undergo maturation, fertilization and early developmental processes. The *HMGB1* expression, as a marker of early development in mice embryos, increased in the groups that treated with low doses of melatonin.

Keywords: Blastocyst. Fertilization. *HMGB1* gene. *In vitro* maturation. Melatonin.

INTRODUCTION

Obtaining high quality matured oocytes is a critical factor for optimal *in vitro* fertilization (IVF) and embryo development (Xu *et al*., 2017). The *in vitro* maturation (IVM) medium is used for maturation of retrieved immature oocytes and increasing the number of matured oocytes that will be used in IVF or the intracytoplasmic sperm injection technique. In addition, IVM is a technique that can be utilized in the patients with the risk of ovarian hyper stimulation syndrome (Jafarabadi *et al*., 2017). Nowadays, IVM is one of the most important techniques in assisted reproduction, although its success rate has been controversial (Teoh, Maheshwari, 2017).

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The reactive oxygen species (ROS) are generated by *in vitro* manipulation and affects both oocytes maturation and embryos development. These free radicals, can damage DNA, ATP synthesis and mitotic spindle that consequently impair the maturation of immature oocyte (Tamura *et al*., 2013). Many studies have reported the harmful effects of ROS on the oocyte maturation and pre-implantation embryo development (Bahadori, Ramezani, Asghari Nohadani 2011; Zhou *et al*., 2016; Dehghani-Mohammad abadi *et al*., 2014-These studies revealed that the protection of embryos from oxidative stresses can improve the embryo development in *in vitro* culture (Leon *et al*., 2004). Many enzymatic and non-enzymatic antioxidants were suggested to reduce the ROS, during the IVM procedure. Some of these components include taurine, hypotaurine, pyruvate, cysteine and glutathione (Zhou *et al*., 2016; Moawad, Tan, Taketo, 2017; Hou *et al*., 2015). In addition, the antioxidant effects of melatonin have been recently proven to be useful for the assisted reproductive technology (Tian *et al*., 2014). Melatonin (N-acetyl-5-methoxytryptamine) is secreted mainly by the pineal gland and has great impact on the reproductive functions of mammals (Tamura *et* al., 2014; Arnao, Hernández-Ruiz, 2014; Dragojevic Dikic *et al*., 2015). Follicular fluid showed a three-fold higher melatonin than the serum in the pre-ovulatory phase (Wang *et al*., 2017). After ovulation, this fluid release to the ampulla of oviduct where the fertilization occurs (Lyons, Saridogan, Djahanbakhch, 2005). Therefore, it is assumed that the melatonin plays a critical physiological role in oocyte maturation and fertilization as well as early embryo development (Cruz *et al*., 2014). In addition, the melatonin represses the expression of *CASP3* and *BAX* genes, which are directly associated with apoptosis. Therefore, the melatonin reduces apoptosis in embryonic cells and increases the development of mice embryo (Dehghani-Mohammad abadi *et al*., 2014). Today, melatonin is widely used for improving the quality of oocytes that were retrieved from infertile women and matured in *in vitro* (Tian *et al*., 2014).

The high mobility group box-1 (*HMGB1*) gene has multiple functions in DNA transcription and repair, differentiation, extracellular signaling and inhibition of apoptosis (Lee *et al*., 2014; Ugrinova, Pasheva, 2017). The expression of *HMGB1* in mice embryos promotes embryonic development to the blastocyst stage through suppressing the expression of *CASP3* and *BAX* genes and consequently reducing the apoptosis in blastomeres of embryos (Cui, Shen, Kim, 2017). As previously mentioned, melatonin has multiple protective effects including oocyte maturation, IVF and embryo development, reducing apoptosis and enhancing embryonic development. However, the effect of melatonin on the expression of *HMGB1* has not been evaluated yet. Regarding the effect of antioxidants on ROS production, the expression level of HMGB1 differs in different tissues. For instance, one study reported that antioxidants reduced the expression of *HMGB1* in pancreas (Tang *et al*., 2007). In contrary, it has been shown that oxidants induced the expression of HMGB1 in lymphatic tissues, especially in macrophages and monocytes (Kang *et al*., 2011). Also a recent study showed that melatonin enhanced the expression of *HMGB1* in *in vitro* matured oocytes (Salimi *et al*., 2014). Therefore, this study evaluated the effect of melatonin on the IVM, IVF, cleavage and blastocyst formation rate of immature mice oocytes and also on the expression of *HMGB1* in blastocysts.

MATERIAL AND METHODS

All chemicals were purchased from Sigma Chemical Corporation (St. Louis, MO, USA) except where noted otherwise. The experiments on animal were approved by the Institutional Animal Care at the Shahid Beheshti University of Medical Sciences (Tehran, Iran). The B6D2F1 mice were procured from the Pasteur Institute of Iran and maintained in temperature and humidity-controlled rooms under 12 hours dark and 12 hours light cycle.

Oocyte Collection and IVM

Immature oocytes at the germinal vesicle (GV) stage were collected from 6- to 8-week-old BDF1 female mice, 48 hours after injection of 10 IU of pregnant mare serum gonadotropin (PMSG). The mice were scarified by cervical dislocation. The immature oocytes were retrieved 48 hours after PMSG injection and cultured in a tissue cell culture medium (TCM) 199 supplemented with 5% fetal bovine serum (FBS). The GV stage oocytes with complete granulosa cell layer were released by puncturing ovarian antral follicles with a 28G needle. The immature oocytes with GV and complete granolosa cell layer were chosen and transferred to 50 μL droplets of TCM-199 supplemented with 10% of FBS, 0.2 mM of sodium pyruvate, 2 mM of L-Glutamin, 10 μg/mL of luteinizing hormone, 10 μg/mL of follicle stimulating hormone, 1 μg/mL of 17β estradiol and different doses of melatonin (10^{-6}, 10^{-9}, 10^{-12} M). Afterwards, the oocytes were incubated at 37 ºC in 5% CO_2 for 22-24 hours.

Assessment of Nuclear Maturation

After the IVM period, the oocytes were denuded from cumulus cells using 1 mg/mL hyaluronidase and the pipetting procedure. The nuclear status of oocytes was evaluated by 10 µg/mL Hoechst (33342) under a fluorescent microscope (Olympus, Tokyo, Japan). The oocytes were classified as metaphase I (MI, containing oocytes with a metaphase plate without polar body) and metaphase II (MII, containing a metaphasic plate with polar body) stages.

Measurement of ROS by Chemiluminescence Assay

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione)-based assay is widely used to measure global ROS production in the medium. Briefly, after maturation, the IVM medium of all the groups was collected and centrifuged at 600 g for 7 minutes. Then, 400 µL of the supernatant was removed and mixed with the Luminol (50 mM). Consequently, the mixture was placed in the Luminometer (LKB 953, Wallac Inc., and Gaithersburg, MD, USA) for 15 minutes and the data were expressed as relative light units (RLU/s).

In Vitro Fertilization (IVF)

Mature oocytes were transferred to 100 µL culture droplets of KSOM that supplemented with 15 mg/mL bovine serum albumin (BSA). Next, for sperm preparation, two male BDF1 mice (10-week-old) were sacrificed by cervical dislocation. The vas deferens was cut and immediately transferred to the Ham's F10 medium that supplemented with 5% BSA. After incubation for 15 minutes, the sperm suspension was centrifuged at 3,000 rpm for 3 minutes. The supernatant was discarded and 300 µL Ham's F10 containing 5% BSA added to the pellet. The swim-up procedure was performed at 37 ºC in 5% CO_2 for 45 minutes. After collecting the motile spermatozoa, 5×10^6 sperms were added to the IVF medium containing the mature oocytes and incubated at 37 ºC under 5% CO_2 for 6 hours.

In Vitro Culture

Zygotes were collected and transferred to 30 µL droplets of the culture medium (KSOM supplemented with 4 mg/mL BSA) under mineral oil and incubated at 37 ºC under 5% CO_2 for 5 days. The number of 2, 4, and 8-cell embryos as well as morula and blastocyst were recorded at 24, 48, 72, 96 hours after fertilization.

Differential Staining

Differential staining was performed to determine the number of trophoectoderm cells, inner cell mass (ICM) and total blastocyst cells. Briefly, the zona pellucida of the blastocyst was removed by acid Tyrode's solution and the blastocyst was transferred to the M2 medium containing rabbit anti-mouse serum at the ratio of 1:2 and incubated for 30 minutes. Afterwards, they were washed with M2 medium and incubated (30 min) in 30% guinea pig complement (in M2) medium that containing 10 mg/mL propidium iodide and 10 mg/mL Hoechst H33342. After fixation with ethanol, the blastocysts were mounted on slides with using of glycerol and finally observed by a fluorescent microscope.

RNA Extraction and cDNA Synthesis

The relative transcript levels of the *HMGB1* were determined by real-time PCR. PCR reactions were performed on an applied Bio-Rad thermo cycler. At least two blastocysts were analyzed for each group. The blastocysts were transferred to the bottom of a 0.2 mL Eppendorf tube containing 1.5 µL lysis buffer. Next, 2 µL of poly N and 5 µL water were added to the embryo and placed in a thermal cycler for 5 min at 75 ºC. The tubes were taken on the ice and 9 µL of the reaction mixture consisting of 5x RT buffer, 200u RT enzyme, 10 mM dNTP and 10u RNase inhibitor added to the samples. The amplification program for the reverse transcription step was performed as follows: 10 min at 25 ºC, 15 min at 37 ºC, 45 min at 42 ºC and 10 min at 72 ºC [23]. After the reverse transcriptase reaction, the samples were kept at 4 ºC for 24 h.

HMGB1 Expression by Quantitative PCR

Real-time quantitative PCR was performed to assess the expression of the *HMGB1* (Forward: 5' -AAGTATGAGAAGGA TATTGCTG - 3' Reverse: 5' - CCAACTTATTCATCATCATCATC- 3', Accession number: NM_010439.3) with using of Rotor-Gene Q instrument (QIAGEN). Real-time PCR reactions were carried out in a total volume of 13 µL according to the manuals of the DNA Master SYBR Green I mix (Roche Applied Sciences). The primer concentrations were adjusted to 1 µM for the reaction of each gene. The cycling parameters were 5 sec at 95 ºC as holding time, 40 cycles of 3 min at 95 ºC, 15 sec at 60 ºC and 10 sec at 72 ºC. The amplification reactions was confirmed by melting curve analysis. The *Hprt1* (Forward:

5' - TCCCAGCGTCGTGATTAG - 3'; Reverse: 5' -CGAGCAAGTCTTTCA GT CC - 3', Accession number: NM_013556.2) was used as internal house-keeping gene. Three replications were performed and the mRNA level of each sample was normalized to that of *Hprt* mRNA level. The relative levels of mRNA were analyzed by the REST 2009 Software (QIAGEN).

STATISTICAL ANALYSIS

All statistical analyses were performed with using SPSS 22 Software (SPSS, Chicago, IL, USA). The means of MI and MII oocytes, 2,4 and 8-cell embryos, morula and blastocyst were compared by the non-parametric analysis test (Kruskal-Wallis). The total cells of blastocysts in experimental groups were compared by using the Tukey's HSD post-hoc test. The relative levels of mRNA were analyzed by REST 2009 Software (QIAGEN). The obtained data were expressed as means ± SEM. Statistically significant difference was accepted at $P \leq 0.05$.

RESULTS

Oocyte Maturation

A number of 1255 of immature oocytes were transferred to IVM media that supplemented with different doses of melatonin. It was observed that the number of matured (MII) oocytes was significantly increased in the media that supplemented with 10^{-6} and 10^{-12} M of melatonin (85% and 79%, respectively). The highest maturation rate was achieved at the highest concentration of melatonin (i.e., 10^{-6} M). Although a positive effect was observed on nuclear maturation in the group that supplemented with 10^{-9} M of melatonin, it was not significant compared to the control group (Figures 1 A and B).

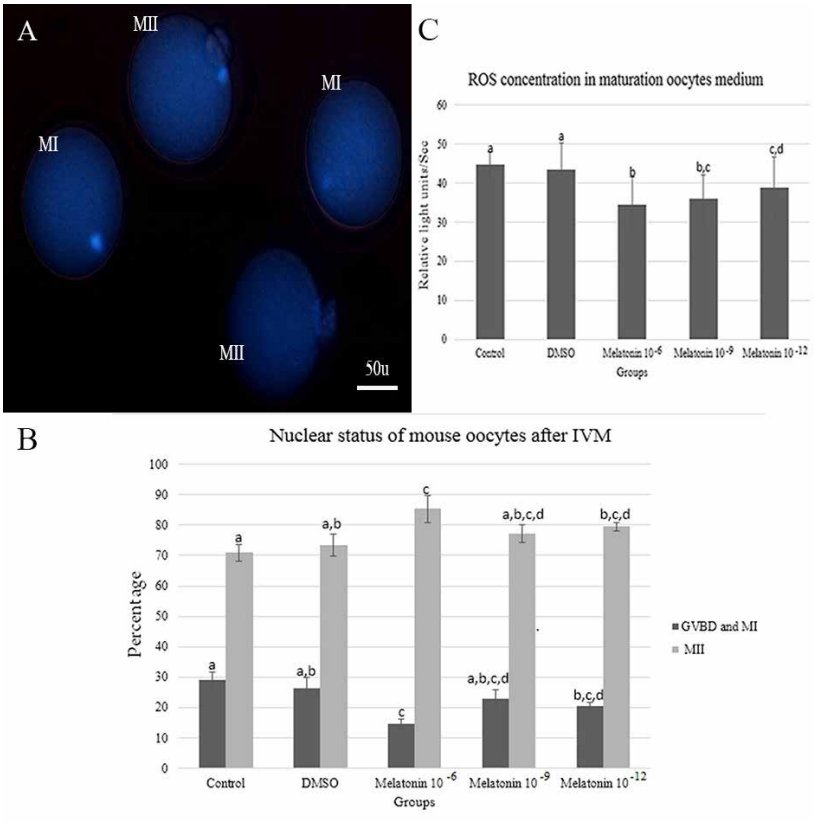

FIGURE 1 – The maturation rate of the oocytes and ROS concentration in culture media:
(A); The fluorescent intensity of Hoechst H33342 stained nuclear mice oocytes after maturation; immature oocytes shown by MI and mature oocytes shown by MII, scale bar 50 µ **(B);** the chart of oocytes maturation (MII) in IVM media containing different doses of melatonin (10^{-6}, 10^{-9}, 10^{-12} M) **(C);** the chart of the ROS concentration in the maturation medium after IVM. The means of MI and MII oocytes were compared by using the non-parametric analysis test (Kruskal-Wallis). ^{a,b,c} Columns with different lowercase letters differ significantly ($P<0.05$). Data were presented as mean ± SEM.

Effect of Melatonin on ROS Concentration

According to the obtained results, the ROS concentration in the media after IVM was significantly different in the oocytes that treated with different doses of melatonin ($p<0.05$). The ROS concentration in the medium that supplemented with 10^{-6} M of melatonin was significantly decreased compared to the others groups, except the 10^{-9} M treatment ($p<0.05$). Moreover, the ROS concentration in the medium that supplemented with 10^{-12} M of melatonin was significantly decreased compared to the others groups, except the 10^{-9} M treatment ($p<0.05$) (Figure 1C). However, the ROS concentration in the medium that supplemented with 10^{-9} M of melatonin was not significantly different with that in the 10^{-6} and 10^{-12} groups ($p>0.05$), but was lower than that in the control and sham groups. Thus, melatonin decreased the ROS concentration proportional to the concentration of melatonin ($p<0.05$).

IVF and *In Vitro* Culture

The rate of fertilization in the medium that supplemented with 10^{-12} M of melatonin obtained 80.9%, which was significantly different with control and other groups ($p<0.05$). The cleavage and blastocyst formation rates in the medium that supplemented with 10^{-12} M of melatonin were significantly improved compared to the control group ($p<0.05$) (Table 1).

TABLE I – Development of the IVF/2-cell mouse embryo cultured in the medium supplemented with different concentrations of melatonin

The means of 2-cell, 4-cell, and 8-cell embryos as well as morula and blastocyst were compared by the non-parametric analysis test (Kruskal-Wallis). Data are presented as mean ± SEM. [a, b, c,d] Numbers with different superscript letters in the same column differ significantly ($P<0.05$).

Group	Oocyte number	2-cell stage N(% ±S.D)	4-cell stage N(% ± S.D)	8-cell stage N(% ± S.D)	Morula N(% ± S.D)	Blastocyst N(% ± S.D)
Control	151	92 (60.90±3.9) [a]	53 (61.75±7.1)[a]	52 (60.63±7) [a]	37 (44.76±9.5)[a]	22 (24.76±4.49) [a]
DMSO	165	103 (63.15±2) [a]	83 (76.96±3.2) [b,c]	70 (67.8±2)[a]	56 (53.86± 3.41)[a,b]	37 (35.12±5) [a,c]
Melatonin 10^{-6} M	247	163 (63.65±3.55) [a]	125 (77.4±7.6) [c]	109 (65.14±6.1) [a.]	100 (60.1±4.4)[b]	79 (48.14±5.14) [b,c]
Melatonin10^{-9} M	229	113 (60.22±2.57) [a]	79 (74±4.3)[d,b,c]	77 (72.88±5.4) [b]	56(53.05±4.39)[a,b]	45 (40.96±7.72) [c]
Melatonin 10^{-12} M	213	171 (80.91±6.36) [b]	51 (86.36±5.37)[e,c]	149 (84.14±9.61)[c]	129(69.5±18.48)[c,b]	105 (53.88±21.27)[d,b]

ICM, Trophectoderm (TE) and Total Cell Number of Blastocysts

Differential staining was performed to determine the cell number of ICM and TE as well as the total cell number of blastocysts (Figure 2, A). The group that supplemented with 10^{-12} M of melatonin showed a significant increase in cell number of ICM and total cell number ($p<0.05$) compared to the control group. However, there was no significantly difference between the groups in terms of trophectoderm cells (Figures 2 A and B).

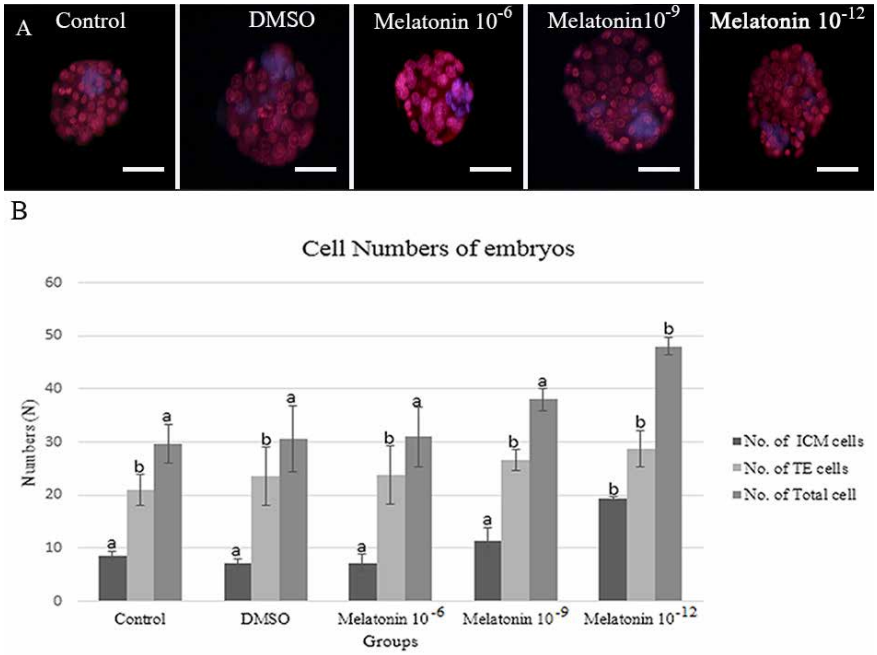

FIGURE 2 – The fluorescence micrograph of differentially labeled mouse blastocysts and chart of the cell number of embryos: (A); the fluorescence micrograph of a differentially labeled mouse blastocyst showing trophectoderm nuclei stains red with propidium iodide and inner cell mass nuclei stains blue with Hoechst H33342 in different doses of melatonin (10^{-6}, 10^{-9}, 10^{-12} M), scale bar 50 μ **(B);** the chart of cell number, ICM and TE in different groups. Total cells of blastocyst in experimental groups were compared by using the Turkey's HSD post-hoc test. [a,b,c] Columns with different lowercase letters differ significantly ($P<0.05$). Data were presented as mean ± SEM.

HMGB1 Gene Expression

The expression level of the *HMGB1* was analyzed by real-time PCR in blastocysts of all the groups. The *HMGB1* expression decreased in the groups that supplemented with 10^{-6}M and 10^{-9}M of melatonin and DMSO compared to the control group ($p\leq0.05$). The *HMGB1* expression in the group that supplemented with 10^{-12} M of melatonin increased compared to the control group, but did not show a significant difference ($p>0.05$) (Figure 3).

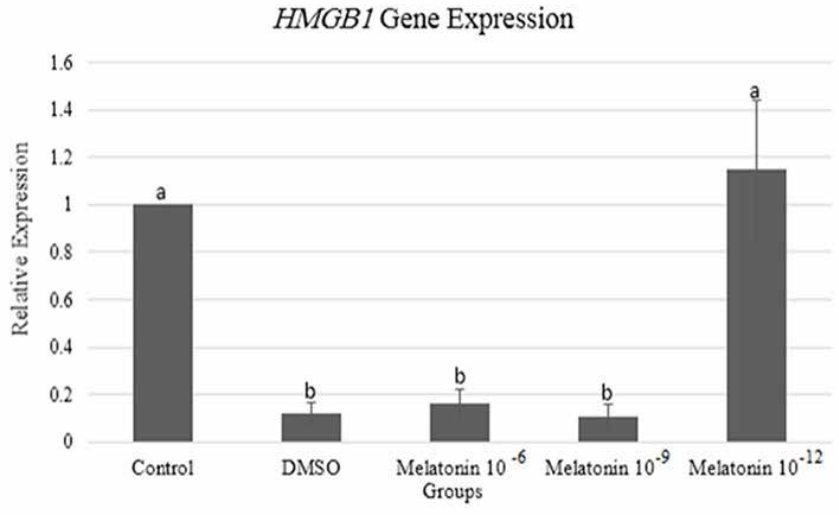

FIGURE 3 – Relative expression levels of the *HMGB1* gene expression in mice blastocysts
Relative expression levels of the *HMGB1* gene in blastocysts cultured with different doses of melatonin and without melatonin. The mRNA levels of *HMGB1* were analyzed with real-time PCR and mRNA levels were normalized to *Hprt* mRNA level. The relative levels of mRNA were analyzed by REST 2009 Software (QIAGEN). [a, b] Numbers with different lowercase letters differ significantly ($P<0.05$). Data were presented as mean ± SD.

DISCUSSION

The components of the culture medium, including proteins, hormones and antioxidants play important roles in the maturation of oocytes and development of the early embryos (Rizos *et al*., 2002). Previously, melatonin was used as an antioxidant for the improvement of oocyte maturation, fertilization and cleavage. However, the most effective dose of melatonin for optimal oocyte maturation in an IVM medium has not been determined so far. (Sirard, 2001). Therefore, this study was designed to evaluate the effects of melatonin on nuclear and cytoplasm maturation of the oocytes.

In this study, it was revealed that melatonin decreased the ROS concentration, proportional to the concentration of melatonin. The ROS concentration in the media with 10^{-6} and 10^{-12} M of melatonin was lowest and highest in all the groups, respectively. Furthermore, our results revealed that all the three doses of melatonin enhanced nuclear maturation by optimization of the ROS concentration. Interestingly, in the groups that were treated with 10^{-6} and 10^{-12} M of melatonin, the nuclear maturation was significantly increased comparing to the control group. These results are in agree with the previous studies showing that melatonin significantly increased the maturation and fertilization rate of mouse immature oocytes in micro molar concentrations (Farahavar, Shahne, 2010; Seo, So, Hyun, 2016; Mokhber *et al.*, 2016). The rate of fertilization depends on the quality of oocytes maturation. It has been shown that the fertilization rate of *in vitro* matured oocytes is lower than that of *in vivo* matured oocytes (Bahadori, Ramezani, Asghari Nohadani, 2016). The cleavage rate and embryonic development in the blastocyst stage were significantly higher in the group supplemented with 10^{-12}M of melatonin comparing to the control and other groups. These results are in contrast to previous report by Bahadori, Ramezani, Asghari Nohadani, 2016 who showing the 10^{-9} M dose of melatonin was more suitable for oocytes maturation, fertilization and cleavage (Bahadori, Ramezani, Asghari Nohadani, 2016). These results suggested that high doses of melatonin increased the maturity of oocytes while higher fertilization and cleavage rate detected in low doses of melatonin. Therefore, it should be considered that low levels of ROS (produced by high dose of melatonin) promote the meiosis of oocytes and release the first polar body while high levels of ROS (produced by low dose of melatonin) give rise to fertilization and cleavage rates. Consequently, medium supplemented with 10^{-12}M of melatonin have optimum ROS levels to induce the oocytes maturation, fertilization and development of embryos.

There are two methods for evaluation of blastocyst quality, first, the total cell number of blastocysts and ICM. In a previous study, it was shown that adding 10^{-9} M of melatonin to the culture medium caused an increase in total cell number of mice blastocysts (Tian *et al*., 2010). The present study showed that supplementation of the medium with 10^{-12} M of melatonin increased the fertilization and cleavage rate, as well as ICM and total cell number of blastocysts. The second method to evaluate the quality of *in vitro* embryos is gene expression analysis. The *HMGB1* gene is a transcription factor that repairs DNA and suppresses apoptotic genes expression (*TRIL*, *BAX* and *Casp8*) (Liu *et al*., 2007).

Furthermore, the *HMGB1* inhibits the signaling pathway of the *P53*. According to the above criteria, the expression of the *HMGB1* can decrease the mortality of embryos and, consequently, improve the development of embryos and blastocyst formation (Cui, Shen, Kim. 2001). According this report, there is a variable expression of the HMGB1 during embryo development, so that a high and low level of expression in the zygote and 2-cell stage was seen respectively. In the final step, the *HMGB1* increased again in the morula and blastocyst stage. They concluded that when the expression of the *HMGB1* increased in embryos, the proliferation and development of embryos were significantly improved. On the other hand, the increased expression of the *HMGB1* in *in vitro* matured oocytes occurred in the presence of 10^{-12} M melatonin. (Salimi *et al*., 2014). Their results are in disagree with previous report which shows the antioxidant, increased the *HMGB*1 expression (Tang *et al*., 2007). However, this paper was the first study to evaluate the expression level of *HMGB*1 in blastocysts (obtained from *in vitro* matured oocytes) that treated with different doses of melatonin. According to the results of this study, the expression level of the *HMGB1* significantly decreased in the blastocysts that treated with 10^{-6} and 10^{-9} M of melatonin. These results are in agree with the previous studies on non-embryonic tissues which revealed a decrease in the *HMGB1* expression in response to high-dose antioxidants. The optimal antioxidant activities are induced by low-dose of melatonin as it leads to increased expression level of the *HMGB1* as well as efficient fertilization, cleavage and good-quality embryo. In this study, the expression level of the *HMGB1* increased in the group that treated with 10^{-12} M of melatonin comparing to the other groups, but did not show a significant difference with the control group. The increasing of the *HMGB1* in

blastocysts that treated with 10^{12} M of melatonin correlated with, high fertilization, cleavage and blastocyst formation rate as well as good embryo quality. Therefore, it can be found that low-dose of melatonin decrease the ROS production and increases the expression of *HMGB1* that positively affects the development of embryos. However, high dose of melatonin highly decreases the ROS level that is not suitable for the *HMGB1* expression. An optimal amount of ROS is required for development, fertilization and expression of the *HMGB1*, which is provided at the concentration of 10-^{12} M of melatonin. Oxidative stress caused by ROS does not always have a negative impact on embryo quality. An efficient concentration of ROS is required for the induction of anti-apoptotic genes and embryo development. The optimal antioxidant effect of melatonin can be induced in low-dose and led to increase the *HMGB1* expression as well as to fertilization, cleavage and good quality embryo. As mentioned before, there is a direct relationship between the cleavage and development of embryos with the expression level of the *HMGB1*. Therefore, it can be assumed that low-dose of melatonin provide the optimal ROS level, which is required for proper expression of the *HMGB1* and high embryo development.

CONCLUSION

The oxidative stress (ROS) in the medium does not always have a negative effect on embryos development. An efficient concentration of ROS is required for the induction of anti-apoptotic genes and embryos development. Low-dose of melatonin leads to optimal antioxidant effect, increasing the *HMGB*1 expression and also the improvement of fertilization, cleavage and good quality embryo.

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CONFLICT OF INTEREST

The authors declare no competing interests.

NOTES ON CONTRIBUTORS

MS designed the experiments; MM and MS performed the experiments; SHP analyzed the data; NS, AJ, MS, and MM contributed reagents/materials/analytical tools; and MS, MN wrote the manuscript. All the authors approved all the revisions and the final paper.

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