Original Article

Melatonin Impact on In Vitro Development of Mouse Preantral Follicles and Oocyte Maturation

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Abstract

Introduction: Melatonin acts as an indirect antioxidant and is a powerful direct free radical scavenger and direct responses to melatonin in the gonads are detected. This study aims to investigate the influence of different doses of melatonin on preantral follicle development and oogenesis of in vitro cultured mouse ovarian follicles.

Materials and Methods: Preantral follicles with diameters of 150–175 μm were mechanically isolated from NMRI mouse ovaries. Follicles were cultured in droplets of α-minimal essential medium (α-MEM) supplemented with 5% FBS, 100 mIU/ml rhFSH, 1% ITS, 100 IU/ml penicillin and 100 μg/ml streptomycin in conjunction with varying doses of melatonin (0, 1, 10, 100 nM and 100, 500 pM) for six days. On day six, in vitro ovulation was induced by the addition of hCG/rEGF to the culture medium and after 16-20 h the maturation state of the oocytes was assessed.

Results: There was a significant ($P<0.05$) decrease in the number of surviving follicles in the groups that received 10, 100 nM and 500 pM melatonin compared to the other groups. After induction of in vitro ovulation, follicles in groups that received 1, 10, and 100 nM melatonin had higher ovulation rates ($P<0.05$) compared with the other groups. Oocyte maturation capacity was adversely influenced by five concentrations of melatonin and GV arrest was significantly higher compared to the control group ($P< 0.01$).

Conclusions: Our data indicates that a dose of 100 pM melatonin has no toxic effects on follicular development and can be used to reduce oxidative stress in follicle culture systems.

Keywords: Ovarian follicle, Melatonin, Ovulation, Oocytes

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Introduction

Although mammalian ovaries contain numerous follicles only a very limited number of them undergo maturation in vivo. In vitro culture of follicles that promotes follicular development accelerates the maturation of large numbers of healthy oocytes. Follicle culture and in vitro maturation (IVM) are tentative techniques to preserve oocytes in patients with premature ovarian failure and is a way to avoid the side effects of hormonal stimulation protocols in some patients who undergo assisted reproduction techniques [1]. The quality of oocytes developed in vitro remains lower compared with in vivo matured oocytes [2-4]. Lower quality of in vitro matured oocytes has been related to the inadequacy of the in vitro environment [5]. An important factor that affects the IVM process in mammals is the culture medium used for oocyte maturation. Composition of the IVM medium influences the developmental capacity of oocytes and embryos [6]. The in vitro environment exposes oocytes and embryos to an excess of reactive oxygen species (ROS) that are not normally produced during in vivo processes. Although ROS are required for various physiological pathways necessary for reproduction, in vivo levels of these molecules are controlled by antioxidants that scavenge and neutralize free radicals to maintain an optimal physiologic oxygen tension in the reproductive system [7].

Melatonin or 5-methoxy-acetyl-tryptamine has an important role in the regulation of electron transfer and detoxification of free radical intermediates [8]. This molecule selectively detoxifies ROS in vitro [9] and is a potent, broad spectrum antioxidant [10-12]. Melatonin is an endogenous hormone produced by the pineal gland, as well as the gut and bone marrow cells or the enterochromaffin cells of the gastrointestinal tract [13,14]. It is also produced in numerous tissues such as the ovaries, testes, vascular system, intestines, and smooth muscles. In addition, some immune cells possess membrane and nuclear melatonin receptors [15,16]. According to research, a direct effect of melatonin on oocytes has been observed [17]. This molecule easily crosses all cellular membranes and enters the cytosol, mitochondria and nuclei [18,19]. Some actions of melatonin are mediated through specific membrane receptors, whereas other functions seem to rely on nuclear binding sites [20]. Although the physiological roles of melatonin in follicular fluid have not been understood, it is possible that melatonin is the most effective antioxidant in the follicle [21].

In the present study we investigate the effect of different doses of melatonin on the development of mouse preantral follicles and their oocytes in vitro.

Materials and Methods

Animals

Female NMRI mice used in this study were housed in a room with a controlled temperature (23-25ºC) and 12 h light:12 h dark cycle. Mice were fed with pellet food and water ad libitum. All study procedures were approved by the Medical Ethics Committee Guilan University of Medical Sciences, Rasht, Iran.

Isolation of preantral follicles

Immature female mice (18–22 day-old) were killed by cervical dislocation. The ovaries were removed and placed in droplets of α-minimal essential medium (α-MEM; Gibco, Invitrogen) supplemented with 10% FBS (Sigma, Germany), 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma, Germany). Preantral follicles were mechanically dissected using 26-G needles attached to a 1 ml syringe under a stereo microscope (Olympus). Follicles with diameters of 150-175 µm and normal appearance (central and spherical oocyte, high density of granulosa_cells and an intact basal lamina) were selected (Fig. 1A).

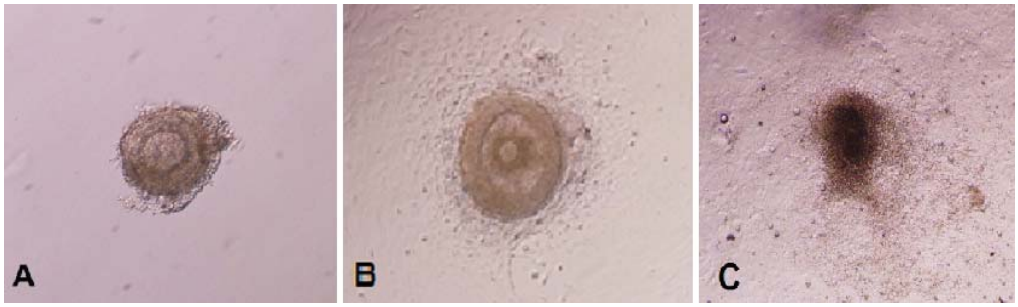

Figure 1. Preantral follicle at the start of the culture time (200x) (A): Preantral follicle with antral-like cavity at day six (40x) (B): Degenerated preantral follicle (40x) (C): Control group.

Follicular diameters were measured under an inverted microscope using a calibrated digital camera.

Culture of preantral follicles

Follicles were individually cultured for six days in 20 µl droplets of α-MEM medium supplemented with 5% FBS, 100 mIU/ml rhFSH (Gonal-f, Merck Serono, Switzerland), 1% ITS (Gibco, Invitrogen), 100 IU/ml penicillin and 100 µg/ml streptomycin covered with mineral oil at 37°C in a humidified atmosphere of 5% CO_2.

In vitro ovulation induction

On day six of culture, 5 ng/ml rEGF (Sigma, Germany) and 1.5 IU/ml hCG (Choriomon, Switzerland) were added to the culture medium of the surviving follicles to induce in vitro ovulation. After 16–20 h of incubation, we checked the follicles for ovulation. Ovulated oocytes were denuded by gentle pipetting from the cumulus cells. A stereo microscope (Olympus) was used to grade the state of the oocytes' nuclear maturation.

Experimental groups

To evaluate the effect of melatonin on follicular development and oocyte maturation, we cultured the follicles in groups of the following melatonin concentrations: 100 pM, 500 pM, 1 nM, 10 nM, and 100 nM.

Evaluation parameters

On day six of culture, we assessed the number of surviving follicles. After 16–20 h, the surviving follicles that had been induced to ovulate were checked for ovulation. The diameters of the oocytes and zona pellucida (ZP) were measured at 200x magnification by a precalibrated digital camera under an inverted microscope.

Statistical analysis

We used the chi-square test to analyze survival and ovulation rate of the follicles, and the nuclear maturation of the oocytes. Oocyte and ZP diameters were analyzed by one-way ANOVA. Data analysis was performed using SPSS 16 software.

Results

Follicle survival

Figure 2 shows the effects of five concentrations of melatonin on follicle survival during the culture period. On day six there was a significant decrease in the number of surviving follicles in those groups treated with 10 nM, 100 nM and 500 pM compared to the control group ($P<0.05$). There was no significant

difference between the control, 1 nM and 100 pM groups (Figs. 1B and C).

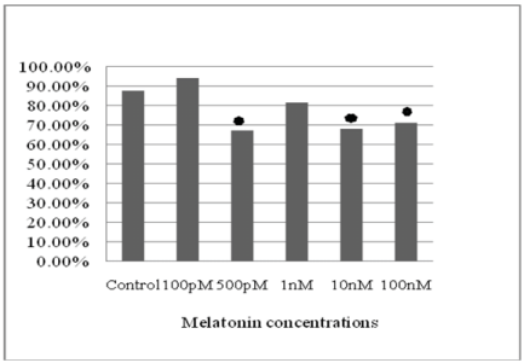

Figure 2. Percentage of surviving follicles in experimental groups at the end of the culture period.
* P<0.05 according to the chi-square test.

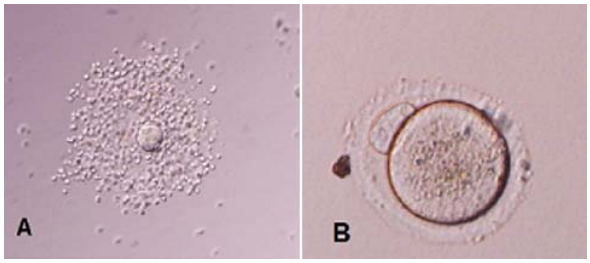

Figure 3. Ovulated COC (100x) (A): Metaphase II oocyte (200x) (B): Control group

In vitro ovulation and cumulus expansion

After induction of in vitro ovulation, the surviving follicles released mucified cumulus-oocyte complexes (COCs; Fig. 3A). When compared with the control group, the 1, 10 nM and 100 nM groups showed a significant increase in the number of COCs (P<0.05). There was no significant difference between the other groups and the control group (Fig. 4).

Maturation stage of oocytes

We observed the highest number of mature oocytes in the 100 pM group (Fig. 3B), however this number was significantly less when compared with the control group (P< 0.01; Fig. 5).

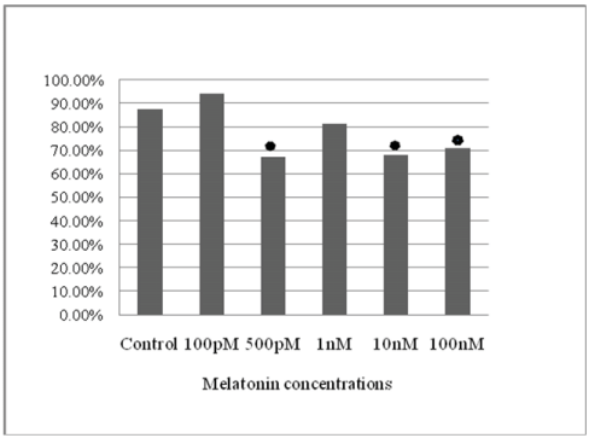

Figure 4. Percentage of COCs in the experimental groups.
* P<0.05 according to the chi-square test

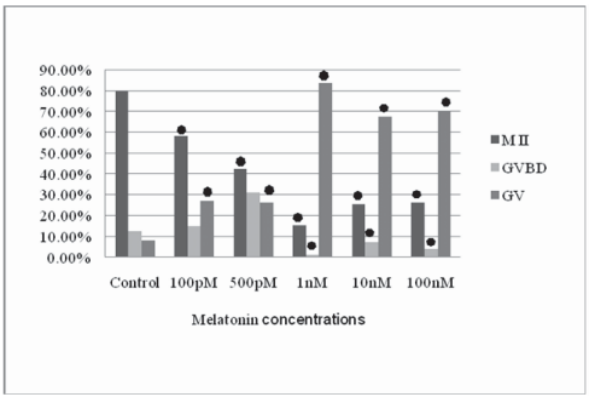

Figure 5. Effect of melatonin on oocyte maturation. M II: Metaphase II oocyte, GVBD: Germinal vesicle break down. GV: Germinal vesicle
* P<0.05 according to the chi-square test

Oocyte and zona pellucida (ZP) diameter

The mean diameter of oocytes in the control group was 70 µm. This diameter decreased with exposure to different concentrations of melatonin (P<0.05). Oocytes in the 10 nM group had the lowest diameters, whereas the highest diameter

observed for the ZP was in the 1 nM group, which was significant (Table 1).

Table 1. Mean diameters ± S.E. of oocytes and zona pellucida (ZP) in the experimental groups.

Melatonin concentrations	Mean diameter (µm) of oocytes	Mean diameter (µm) of zona pellucida (ZP)
100 nM	66.52±0.8[a]	8.42±0.2
10 nM	63.18±0.5[a]	7.33±0.1
1 nM	67.21±0.7[a]	9.85±0.2[a]
500 pM	63.03±0.5[a]	7.06±0.1[a]
100 pM	66.91±0.5[a]	7.76±0.1
Control	70.19±0.7	8.07±0.2

[a] P<0.05 according to ANOVA

Discussion

Oxidative stress can alter cellular molecules such as lipids, proteins and nucleic acids. Oocytes and embryos produced in vivo can be protected against free radicals by antioxidants that exist within the follicular and oviduct fluid [22]. Melatonin plays a key role in a variety of important physiological functions. Some effects of melatonin are mediated by membrane receptors but many of them exert direct free radical scavenging properties, a process that requires no receptor [21]. Physiological melatonin concentrations in human blood seems to be in the range of 100 pM to 1 nM; in follicular fluid, it is three times higher than in serum at the same time [23-25].

It has been demonstrated that the efficacy of exogenous melatonin in modifying particular reproductive functions varies among species according to age and timing of its administration [26].

The results of our study showed that high concentrations of melatonin reduced the number of surviving follicles. Morphological observations of degenerated follicles showed a high reduction in the number of granulosa cells. The effect of melatonin has been shown by Adriaens et al., where follicle survival decreased when 2 mM of melatonin was added to the culture medium [27]. In pinealectomized female Syrian hamsters, melatonin administration has been shown to inhibit in vivo follicular development [28].

Melatonin's inhibition of cell proliferation has been reported in numerous cellular models [29]. It has been shown that in cultured Chinese hamster ovarian cells, high doses of melatonin caused decreased cell numbers [30].

High levels of ROS can damage spermatozoa, oocytes and embryos [31]. The presence of melatonin in follicular fluid and its receptors in granulosa cells indicate the important role of melatonin in reproduction [24,32]. It has been demonstrated that a melatonin concentration of 10^{-6} M significantly increased the maturation rate of sheep oocytes, however a 10^{-5} M concentration of melatonin in culture medium did not significantly influence maturation, fertilization, or cleavage [33]. Kang et al. reported that supplementation of culture medium with 10 ng/ml melatonin during IVM of porcine oocytes compared with the control group increased the proportion of oocytes that extruded polar bodies [32].

Our study showed a significant decrease in oocyte maturation rate in all doses of melatonin that was associated with a reduction in oocyte diameter. Wang et al. demonstrated that melatonin in cultured COCs inhibited the formation of the first polar body, but had no effect on germinal vesicle break down. In addition, melatonin inhibited the effects of FSH on resumption of meiosis [34]. In another study supplementation of bovine COC culture medium with 10^{-9} M melatonin, alone or in combination with gonadotropins, did not affect nuclear maturation, nor did it affect the cleavage and blastocyst rates [35]. In addition

it has been shown that melatonin in the culture medium did not improve cumulus cell expansion and nuclear maturation of bovine oocytes [36]. Although melatonin toxicity is reported to be extremely low, oocyte maturation in female mice was significantly impaired by melatonin concentrations of 10^{-3} M or higher [33].

In this study the reduction of oocytes diameter correlated with the oocyte maturation state. It was demonstrated that bovine oocytes with an inside-zona diameter less than 95 μm were unable to resume meiosis in vitro [37]. The ability of bovine oocytes of different sizes to mature in vitro was also investigated by Fair et al. [38]. Hyttel et al. and Sirard et al. have also shown that oocytes gradually acquire competence to undergo meiotic maturation and sustain embryonic development after reaching a diameter between 110 and 120 μm [39].

ROS in nontoxic levels act as signaling molecules. They play a role in the balance between cell growth and death [40]. In many cell lines such as MCF7 and hepatoma AH130, physiological levels of melatonin delay cell progression from the G1 to the S-phase, prolonging the total duration of the cell cycle [23]. It is possible that melatonin influences all cell cycle phases by modifying several cell events, given the fact that cells have not only stopped in the G0/G1 phase, but some also stopped in the G2 gap [30]. In conclusion this study has demonstrated that supplementing of culture medium with 100 pM melatonin decreases oxidative stress in mouse follicles. High doses of melatonin have toxic effects on the follicles.

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