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Molecular, Biological, Physiological and Clinical Aspects of Melatonin

Melatonin-related genes expressed in the mouse uterus during early gestation promote embryo implantation

Abstract: Melatonin, a superior antioxidant, is an important molecule which regulates female reproduction due to its receptor-mediated and receptor-independent antioxidant actions. In this study, we investigated the effect of melatonin on early gestation in a mouse model. During early gestation, the expression of the melatonin's rate-limiting enzyme, *AANAT*, gradually increased – in the uterus while the *MT2* melatonin receptor was only expressed at day 2 of gestation and no *MT1* was detected. Based on these findings, we conducted a melatonin injection experiment which demonstrated that 15 mg/kg melatonin significantly improved the number of implantation sites and the litter size. Also, the blastocyst and uterus were collected to identify the local action of melatonin. In the melatonin-treated mice, the endometrium was thicker than in the control mice; melatonin also caused an increase in density of uterine glands, and the uterine gland index (UGI) was significantly elevated over that of the control. Serum steroid hormone measurements revealed that at day 6 of gestation (postimplantation), melatonin significantly downregulated the E_2 level, with no obvious effects on progesterone. Gene expression assay revealed that melatonin significantly upregulated expression of *HB-EGF*, a crucial gene involved in implantation as well as its receptor *ErbB1* in the blastocyst. In addition, *PRA*, an important gene which influences the decidual response and luminal cell differentiation, *p53*, which regulates uterine through leukaemia inhibitory factor (LIF), were both increased after melatonin treatment. These data suggest that melatonin and its MT2 receptor influence early gestation. Exogenous melatonin treatment can improve mouse embryo implantation and litter size, which may have important applications in human reproductive health and animal husbandry.

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Introduction

In mammals, the fusion of the sperm and ovum normally leads to a live birth [1]. To develop normally, however, the zygote needs to undergo several critical steps: early embryo development, embryo implantation and postimplantation fetal development. Implantation is a key process and requires a series of changes leading to effective reciprocal signaling between blastocyst and uterus, which is regulated by ovarian estrogen and progesterone [2, 3].

From the clinical point of view, implantation failure is a major reproductive disorder [4, 5]. The normal implantation process is divided into three successive phases termed apposition, attachment, and penetration; these are tightly regulated by implantation-related genes, including *HB-EGF*, *P53*, *ErbB1,* and *COX1/2* [6–9]. Apposition is the primary adhesion between the blastocyst and endometrial surface; at this stage, the trophoblast contacts the luminal epithelium closely [10, 11]. Following apposition, the trophoblast and the luminal epithelium form a strong connection which resists dislocation of the embryo. Following adhesion, the blastocyst penetrates the epithelial tissue and passes into the stroma to establish an intimate relationship with the maternal vasculature [12]. These three events must be completed in a restricted time frame termed the 'implantation window' in which the endometrium is receptive to the blastocyst. In mice, the 'implantation window' occurs approximately at day 4 of gestation [13, 14].

Melatonin (*N*-acetyl-5-methoxytryptamine) is a circadian hormone with powerful antioxidant functions; melatonin originates from the pineal gland and other cells. Besides melatonin, its metabolites, including AFMK and AMK, also have the ability to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) [15–18]. In addition to its antioxidant functions, melatonin has an important role in regulating the physiology of both plants and animals [19–22]. In animals, melatonin is involved with the regulation of biorhythms and control of seasonal reproduction in photoperiodic animals [23–25]. In theory, every cell may possess the ability to synthesize melatonin given that it is believed to be produced in mitochondria [26, 27]. In addition to the pineal gland, melatonin is produced in peripheral tissues where it seems to function in regulating local physiological processes [28–30].

Numerous studies have investigated the function of melatonin in female reproduction. The level of melatonin in

human follicular fluid significantly exceeds that in simultaneously collected blood samples. Melatonin in follicular fluid exhibits a 24-hr rhythm and reportedly increases as the follicle enlarges [31–34]. Several studies have demonstrated that exogenous melatonin downregulates estradiol level, while upregulating progesterone in rat [35]; it also promotes oocyte maturation in vitro and enhances implantation of a transferred embryo [36, 37]. Finally, the circadian variations of melatonin in the maternal circulation persist during pregnancy and may regulate the timing of parturition [38–40].

Previous research has shown that melatonin improves the functioning of the ovaries and participates in the process of follicle development including ovulation. To our knowledge, however, no study has tested the functional relationship between melatonin and uterus in mice. Herein, we conducted a series of experiments to determine whether melatonin influences the development of uterus and embryo implantation. Initially, we examined the distribution of melatonin membrane receptors, *MT1* and *MT2*, and measured the enzyme *AANAT* which controls melatonin synthesis at different ages of gestation; thereafter, we evaluated the effect of melatonin on endometrial development, the number of implantation sites, and litter size. We also measured steroid hormone alterations and expression changes of implantation-related genes (*HB-EGF*, *ERα, PRA*, *P53*, *Ihh*, *COX1, HOAX11*, *ErbB1*, *CB1*) and anti-apoptosis genes (*Caspase3*, *BCL-2*). Also, anti-oxidative genes (*SOD1*, *Gpx-1*) were measured to uncover the molecular mechanism of the function of melatonin during implantation. The findings reveal a previously undescribed action of melatonin, that is, the promotion of embryo implantation, improvement in litter size due to its regulation of the endometrium and uterine glands, uterus receptivity, and blastocyst activation.

Materials and methods

Animals

CD-1 mice (12–16 wk old) were purchased from Vital River Laboratories Co. Ltd. (Beijing, China). Mice were housed under controlled conditions of temperature (22–26°C) and light (12-hr light: 12-hr dark cycle) and had access to food and water ad libitum. All experimental procedures were approved by the animal care committee of the China Agricultural University. To ensure the consistency of gestation time, female mice were mated with male mice at night, and females with a vaginal plug were selected for the study. Mice received melatonin (Sigma, St. Louis, MO, USA) intraperitoneally every 12 hr at doses of either 0.15, 1.5, 15, or 75 mg/kg of body weight which continued until the morning of day 6 (D6) of gestation. The mice were killed at D8 for implantation site count, and litter size and litter weight were determined. The optimal concentration of melatonin was determined by implantation site number and chosen for the subsequent study.

Uterine section and morphometric analysis

Formalin-fixed and paraffin-embedded uteri at the night of D4 (implantation window) from the different groups (15 mg/kg melatonin treated or control) were subjected to routine 5-μm thickness sectioning, and one in every five sections was chosen for hematoxylin and eosin (*H&E*) staining. Thereafter, morphometric measurements were performed using digitalized images obtained directly from the inverted microscope (Nikon, Inc., Tokyo, Japan) through a video camera; then, images were saved as graphic files in TIFF format. A total of 12 sections from each uterus were analyzed. The morphometric parameters were measured using Image Pro-Plus software (Image Pro-Plus 6.0; Media Cybernetics, Silver Spring, MD, USA).

The total number of uterine glands (UGN) per section was counted. The endometrial perimeter (P1) and the inner luminal perimeter (P2) were traced; the area bounded by the latter was subtracted from that bounded by the former (P1–P2), and the result was taken as the endometrial area (EA). The area of each uterine gland also was automatically calculated by software. EA and uterine gland gross area (UGGA) were further used to determine the UGI according to the formula: UGI = UGGA/EA, and uterus gland density (UGD) where the UGD = UGN/EA [35].

Progesterone and estradiol-17β level analyses by radioimmunoassay

Progesterone (P) and estradiol-17β (E_2) concentrations were measured on days 4 and 6 of gestating mice (before and after implantation). Blood of each mouse (melatonin-treated and control group) was collected from caudal vein simultaneously. After clotting for 30 min, the serum was obtained by centrifugation at 4°C (1500 g) for 10 min. The levels of P and E_2 were detected by radioimmunoassays (RIAs). The procedures used for the RIAs were those provided with the directions for the Progesterone Direct RIA Kit (ICN Biomedicals, Irvin, CA, USA) and the Estradiol-17β (E_2) Direct RIA Kit. (ICN Biomedicals). The RIAs involved chromatographic separation of the steroids to enhance specificity. The RIAs used a tracer and a charcoal–dextran method to separate the bound and free steroid. The kits included a specific antibody that had no significant cross-reactivity with other steroids.

Melatonin assay using high-performance liquid chromatography (HPLC)

To evaluate the melatonin level in injected and control mice, the animals were anesthetized with avertin (0.024 mL/g, i.p.; 1.4% tribromoethanol plus 1.4% isoamyl alcohol in water, pH 5.5) at 0, 1, 4, 8, 12 hr after intraperitoneal melatonin injection. Blood was collected from the caudal vein, and serum was obtained by centrifugation as above. The sample preparation and detection were performed as previously described by Zhao et al. [41].

Gene expression assay using reverse-transcriptional PCR or real-time PCR

To test the expression of *MT1*, *MT2,* and *AANAT* at different periods of gestation, the uteri were collected in the morning of D1 through D6; the blastocyst and uteri at D4

were also collected for implantation-related gene assay, and the number of blastocyst per mice was counted. Total RNA was extracted using the TRIzol reagent (Invitrogen Inc., Carlsbad, CA, USA) and immediately reverse transcribed using PrimeScript™ RT reagent Kit with cDNA Eraser (TaKaRa Bio Inc., Tokyo, Japan). RT-PCR was conducted to detect the expression of *MT1* and *MT2*. The reaction procedure was as follows: initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and then extension at 72°C for 30 s. The above procedures were repeated 35 cycles with a final extension at 72°C for 5 min. Qualitative polymerase chain reaction (qPCR) amplification was performed by LightCycler 480 SYBR Green I Master Mix (Roche Applied Science, Mannheim, Germany) on LightCycler 480 II PCR machine (Roche Applied Science). The real-time PCR consisted of 10 μL SYBR Green, 30 μM forward and reverse primers, 2 μL template, and ddH_2O was added to a total volume of 20 μL. The procedure was as follows: 95°C for 10 min; 35 cycles of 95°C for 10 s and 60–62°C for 8–15 s; melting curve from 65 to 95°C, increasing in increments of 0.5°C every 5 s. Normalization was performed using the housekeeping gene actin as a control. Primer sequences are listed in Table 1. Relative mRNA expression was calculated by the $2^{-\triangle\triangle ct}$ method.

Protein localization assay by immunohistochemistry

Formalin-fixed and paraffin-embedded uteri at D2 were subjected to routine 5-μm thickness sectioning for the immunohistochemistry. Sections were deparaffinated by incubating with xylene for 30 min and rehydrated in an ethanol series. Sections were then placed in 0.1 M citrate buffer at a pH of 6.0 for antigen repair in a pressure cooker. After antigen repair, sections were submitted to endogenous peroxidase blockage in 3% hydrogen peroxide for 20 min and then equilibrated in 0.1 M PBS (BIOSS Bio Inc., Beijing, China) at a pH of 7.4. Nonspecific binding was blocked using 5% donkey serum (SC-2044; Santa Cruz Bio Inc., Shanghai, China) for 30 min at 37°C and then incubated with goat polyclonal antibodies (MT2: SC-13177; Santa Cruz Bio Inc., Santa cruz, CA, USA, 1:100 dilution with 5% donkey serum; AANAT: SC-55612; Santa Cruz Bio Inc., 1:100 dilution with 5% donkey serum) overnight at 4°C in a humidified chamber. The sections were then washed five times with PBS (5 min per wash) and incubated with secondary antibody (donkey anti-goat IgG-B: SC-2042; Santa Cruz Bio Inc., 1:200 dilution with PBS) for 1 hr at 37°C. After washing with PBS, sections were incubated with streptavidin–HRP for 2 hr (Cwbiotech Inc., Beijing, China). After rinsing with PBS, target protein was visualized using DAB (Sigma) as the chromogen. The developing time of MT2 was 5 min and AANAT was 2 min. Then sections were counterstained with hematoxylin. Negative controls were prepared using 5% donkey serum instead of the primary antibody solution.

Statistics analysis

Data are expressed as mean ± S.E.M. Statistical analyses were done using the univariate analysis of variance (ANOVA) with the aid of SPSS 19.0 statistical software followed by the Student *t*-test. $P < 0.05$ was considered significant, and $P < 0.01$ was considered highly significant.

Results

A total of 53 mice for quantitative real-time PCR or RT-PCR analysis were used at D1, D2, D3, D4, D5, D6, respectively. The results indicated that the rate-limiting enzyme of melatonin synthesis *AANAT* exhibits upregulated expression during the early gestation period, and the expression level at D2, through D6 was significantly higher than the day of the vaginal plug (D1) (Fig. 1A,B) ($P < 0.01$). The expression of melatonin membrane receptor *MT2* was mainly detected at D2; little or no expression at other times was apparent (Fig. 1C). The other melatonin receptor (*MT1*) was not expressed at any point (Fig. 1D). Immunohistochemistry was used to identify and localize MT2 and AANAT protein in mouse uterus at D2. The results showed that MT2 protein signals were highly detected in the luminal epithelium and uterine glands. AANAT protein was mainly located in luminal epithelium, uterine glands, and minimally in stromal cells (Fig. 1E). These measurements suggested that melatonin may be involved in the regulation of gestation in mice.

It was of particular interest to determine whether melatonin would improve the implantation and litter size of pregnant mice. A functional study of melatonin was performed following the intraperitoneal injection in a total 138 mice. The results showed that the dose of 15 mg/kg melatonin significantly increased the implantation sites (16.0 ± 1.68 versus 14.4 ± 1.91, $P < 0.01$) (Fig. 2A). Consistent with implantation site analysis, 15 mg/kg dose of melatonin also significantly improved the litter sizes (13.9 ± 1.23 versus 12.6 ± 1.39, $P < 0.05$, n = 15) (Fig. 2B), while analysis of litter weight showed no obvious difference between melatonin-treated and control mice (Fig. 2C). In addition, we flushed the blastocysts from uteri at D4; these data showed no difference between melatonin and control animals (Fig. 2D). To further determine the concentrations of melatonin caused by its injection, 15 mg/kg melatonin was given in the peritoneal cavity of experimental mice at different time points and serum was collected for melatonin measurement using HLPC. The data showed that at 1 and 4 hr after injection, melatonin levels reached peaks that were nearly 3.6 times higher than basal levels (6.7 ± 1.26 versus 1.8 ± 0.76, $P < 0.01$, n = 10) (ng/mL); these returned to basal values after 12 hr (Fig. 2E). This result verified that intraperitoneal administration of melatonin was absorbed.

As mentioned above, melatonin increased the implantation sites and litter sizes. To elucidate the mechanisms, we examined the histological structure of the endometrium. The EA, density of uterine glands and UGI were measured using the mentioned software. As expected, the EA in the melatonin-treated mice was much larger than in control animals (0.182 ± 0.0025 versus 0.155 ± 0.0028, $P < 0.01$, n = 10) (mm^2) (Fig. 3A). Melatonin mice showed an increased density of uterine gland (172.8 ± 6.90 versus

Table 1. Primers for RT-PCR and qRT-PCR

Genes	Primer sequence (5′–3′)	Product size (bp)	Tm (°C)
β-Actin	Forward: CCAGCCTTCCTTCTTGGGTAT Reverse: AGGTCTTTACGGATGTCAACG	93	60
MT1	Forward: CCATTTCATCGTGCCTATG Reverse: GTAACTAGCCACGAACAGC	259	58
MT2	Forward: TACATCAGCCTCGTCTGGCTCC Reverse: TTCCTCGTAGCCTTGGCCTTCC	239	58
AANAT	Forward: TGAACATCAACTCCCTGAAACCT Reverse: TTCCCGCTCAATCTCAAACG	262	60
HB-EGF	Forward: GGGCCTCCTGTAATTGCTCT Reverse: GCTCACTCGATCCTGCTTTC	113	60
ErbB1	Forward: AAGGCACAAGTAACAGGCTCA Reverse: CCAAGTTCCCAAGGACCACT	114	60
CB1	Forward: TGCTGTTGCTGTTCATTGTG Reverse: TTGCCATCTTCTGAGGTGTG	124	60
P53	Forward: TGAGGTTCGTGTTTGTGCCTGC Reverse: CCATCAAGTGGTTTTTTCTTTTGC	165	60
Ihh	Forward: CTACAATCCCGACATCATCTTCAA Reverse: CGGTCACCCGCAGTTTCA	140	62
COX1	Forward: ACAGTGCGGTCCAACCTTATC Reverse: ACAGAGGGCAGAATGCGAGTA	113	60
Hoax11	Forward: ATAGCACGGTGGGCAGGAACG Reverse: AGTCGGAGGAAGCGAGGTTTT	96	62
ERA	Forward: TGTCCAGCAGTAACGAGAAAGG Reverse: TGGTAGCCAGAGGCATAGTCAT	94	60
PRA	Forward: TGAGCATTGAGCCTGATGTG Reverse: AAAGCAGTTGTCTCTCGCCTA	108	60
Bcl-2	Forward: ACCTGTGGTCCATCTGACCCTC Reverse: CCAGTTCACCCCATCCCTGA	163	60
Caspase3	Forward: CTGGAGAAATTCAAAGGACGGG Reverse: TGAGCATGGACACAATACACGG	194	60
SOD1	Forward: CACTCTCAGGAGAGCATTCCA Reverse: CCCAGCATTTCCAGTCTTTG	110	60
Gpx1	Forward: CACTCTCAGGAGAGCATTCCA Reverse: CCCAGCATTTCCAGTCTTTG	211	60

136.1 ± 6.32, $P < 0.01$) (per mm^2) (Fig. 3B). Also, the UGI was significantly different between melatonin and control mice (57.7 ± 2.27 versus 49.8 ± 2.46, $P < 0.05$) (Fig. 3B). These data suggested that melatonin improves the development of the gestational uterus (Fig. 3C).

The serum from a total of 113 mice at D4 and D6 was collected for P and E_2 assays. At D6 (postimplantation), melatonin significantly lowered the concentration of E_2 (59.7 ± 1.58 versus 72.0 ± 3.37, $P < 0.01$) (pg/mL), while P exhibited no obvious difference between the groups ($P > 0.05$). In contrast, the levels of E_2 and P were not influenced by melatonin treatment on D4 ($P > 0.05$) (Fig. 4).

To further clarify the potential mechanisms of melatonin's beneficial effects on embryo implantation, the blastocysts and uterine tissues were collected at D4 for gene assays. Implantation-related genes, anti-oxidative genes, and apoptosis-associated genes were examined using qRT-PCR analysis. In the uterus, the expressions of implantation-related genes *HB-EGF*, *P53*, *PRA* were significantly increased in melatonin-treated mice ($P < 0.05$, $n = 6$) (Fig. 5A), and no affect was observed on the expression of *Ihh*, *COX1*, *Hoax11*. In the blastocyst, melatonin injection significantly upregulated the expression of *ErbB1* (also named *EGFR*), but not *CB1* ($P < 0.05$, $n = 6$). Surprisingly, melatonin did not influence the expression of anti-oxidative genes (*SOD1*, *Gpx-1*) or of apoptosis-associated genes (*Caspase3*, *Bcl-2*) (Fig. 5B).

Discussion

Embryo implantation is the first physical interaction between the blastocyst and the uterus. The quality of implantation determines the quality of the subsequent gestation. Any disturbance at this process often contributes to early embryo loss and pregnancy failure [42]. In recent decades, numerous molecules involved in the cross talk of the blastocyst and the uterus during implantation have been identified. Many signaling molecules were found to be crucial for embryo implantation in mice and are of high clinical significance for human female reproductive health [43–45]. Melatonin is a multifunctional agent that is known to influence female reproduction [46–48]. It accomplishes this, in part, through activation of receptor sites within the hypothalamic–pituitary–gonadal axis [49, 50]. Several reports have proven that high concentrations of melatonin are present in preovulatory follicular fluid [32, 51]. The expression *of MT1* receptor was reported to be reduced in mouse ovary after birth [52], while the expression of MT1 receptor was significantly higher in ovarian tissue of the rat sacrificed during proestrus than during metestrus [53]. This infers that melatonin may play

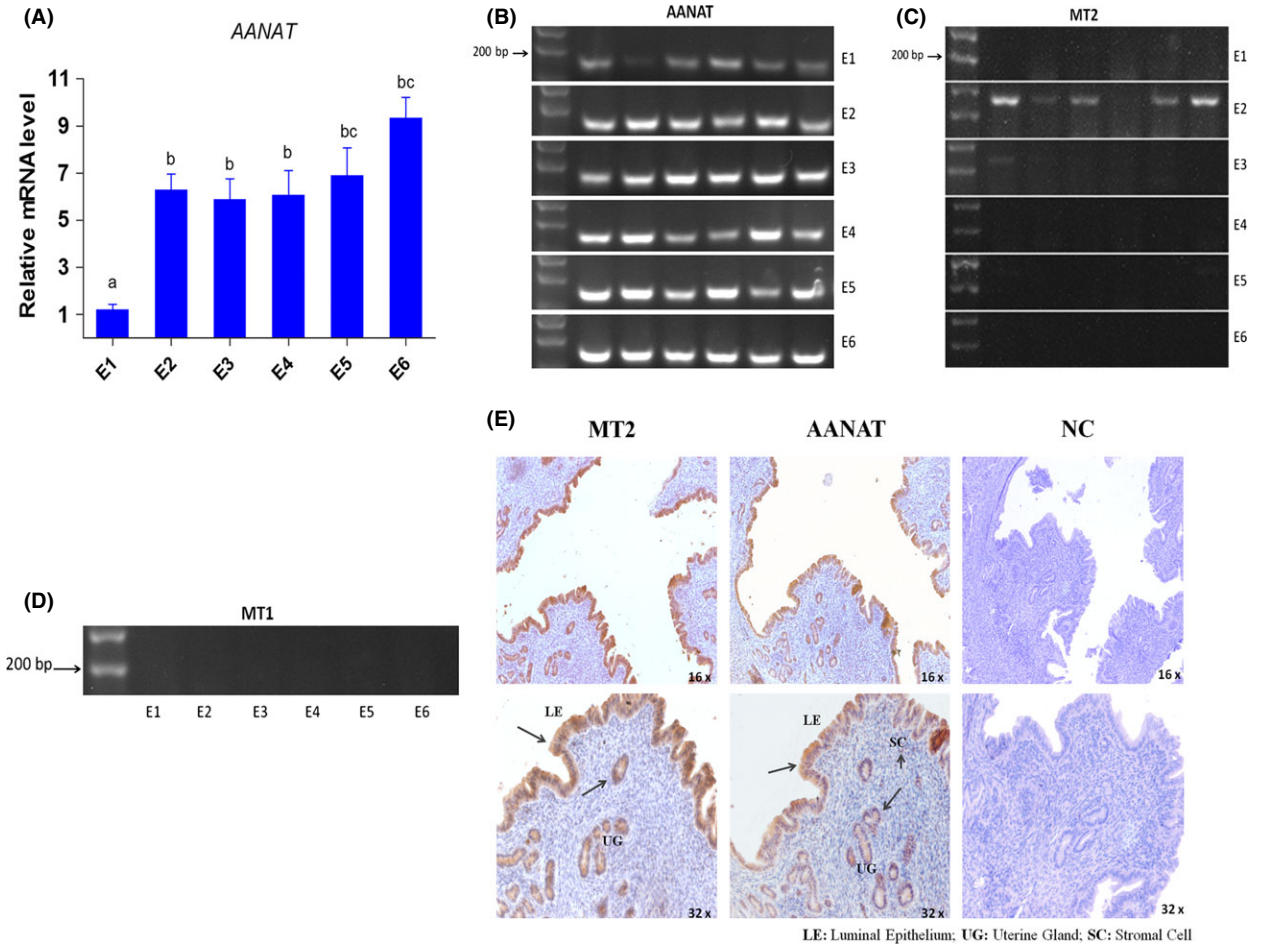

Fig. 1. The expression of *AANAT*, *MT2,* and *MT1* in uterus during the early gestational stage. (A, B) *AANAT* level assayed by qRT-PCR and semi-quantitative RT-PCR, respectively; (C) *MT2* level assayed by semi-quantitative RT-PCR; (D) *MT1* level assayed by semi-quantitative RT-PCR. (E) Location of MT1 and AANAT was determined by immunohistochemistry. A total of 53 mice were used for this experiment. The different superscript letters (a–c) represent a significant difference of these columns ($P < 0.01$).

an important role in folliculogenesis, ovulation, and estrous cycle regulation. In the current study, we confirmed that the expression pattern of melatonin-related genes demonstrates some particular regularity during early gestation. The *MT2* membrane receptor was mainly expressed in luminal epithelial cell and uterine glands of the D2 uterus. The expression of the rate-limiting enzyme *AANAT* gradually increased with the gestation and was mainly localized in luminal epithelial cells, uterine glands, and slightly in stromal cells. These findings are consistent with melatonin being involved in the regulation of early gestation. Contrary to earlier reports in the rat [54], no *MT1* receptor was detected in mouse uterus at D1–D6 (Fig. 1).

To examine the function of melatonin during early gestation in mice, we injected melatonin into the peritoneal cavity of pregnant mice, at a dosage reported in the original study of Wang et al. [55]. The results showed that 15 mg/kg melatonin increased the number of implantation sites and the litter sizes. Also, we validated the feasibility of intraperitoneal injections, with the result that injection with 15 mg/kg melatonin increase serum melatonin levels to nearly 3.6 basal values. We also determined the optimal concentration of melatonin was about 10^{-8}–10^{-9} M (Fig. 2), which is consistent with the previous report of Wang et al. [36, 56], who claimed that 10^{-7}–10^{-9} M melatonin promotes the development of in vitro bovine and mice embryos. A study by Dair et al. [35] showed that pinealectomy significantly reduced the success of rat embryo implantation and supplementation with melatonin reversing these adverse effects to a large extent. Herein, we show that artificially elevating the level of melatonin also promotes embryo implantation in mice.

In mice, implantation occurs on 4 day after mating when the corpus luteum is fully formed [57]. During the interval of preimplantation, the endometrium undergoes a rapid and extensive proliferation of uterine epithelial and stromal cells and becomes receptive to the blastocyst [58–60]. Uterine glands are regarded as the primary source of nutrients for early embryos, and the development state of the uterine glands is directly related to embryo survival and implantation, as well as for the establishment and maintenance of gestation [61–63]. Herein, we show that, after melatonin treatment, the ensuing rise of this hormone had a beneficial effect on the endometrium as well as on uterine gland growth (Fig. 3). Meanwhile, considering the

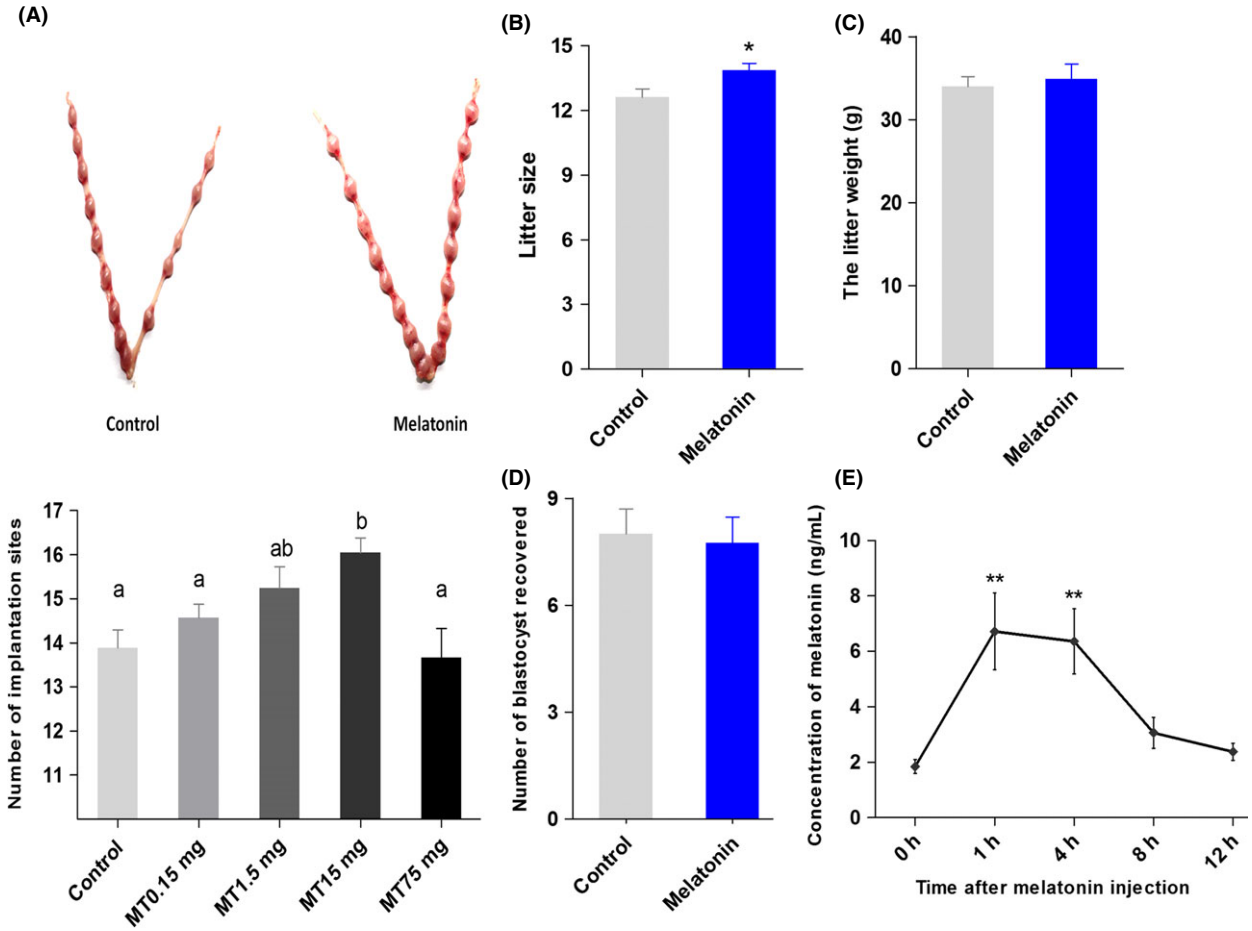

Fig. 2. Effect of melatonin on the number of implantation sites and litter sizes. (A) Number of implantation sites; (B) litter size; (C) the litter weight; (D) number of blastocysts recovered; (E) the variation of serum melatonin levels after melatonin administration. A total of 138 mice were used for implantation site statistics, 30 mice for litter size statistics, 22 mice for blastocyst number statistics, and 50 mice for melatonin assay. The different superscript letters (a, b) represent a significant difference of these columns ($P < 0.05$), *represents significant differences, $P < 0.05$, and **represents significant differences, $P < 0.01$.

expression pattern of *MT2* and *AANAT* during early gestation (Fig. 1), this action of melatonin on endometrium and uterine glands may be an explanation for the improvement of embryo implantation and mediated by MT2.

Previous studies have uncovered evidence that melatonin has a negative influence on E_2 secretion, while elevating the P level [64, 65]. Similarly, despite no changes in P, our data suggest that melatonin can reduce E_2 levels at the postimplantation stage (D6) (Fig. 4). The low level of E_2 likely reduces uterine contractions and the risk of miscarriage. Interestingly, the study showed that on D4 (preimplantation), melatonin was without influence on E_2 secretion. As is known, on day 4 of gestation, a small surge of E_2 is needed to induce mouse uterine receptivity. Indeed, a hypothesis has been proposed that the blastocyst synthesizes and secretes E_2 locally to initiate implantation [43, 44]. We speculate that on day 4, an unidentified mechanism in the blastocyst actively upregulates the level of E_2 to sensitize the uterus; this action is not affected by melatonin.

It has been reported that melatonin significantly promotes in vitro blastocyst rate in the mouse and in bovine [36, 56]. In the current study, nevertheless, no obvious change was detected in the number of blastocysts recovered after melatonin treatment (Fig. 2D). Thus, we further studied the effect of melatonin on blastocyst activation and endometrial receptivity by analyzing the changes in implantation-related genes. Blastocyst activation implies the acquisition of implantation competency which is a prerequisite for successful implantation and regulated by numerous signaling molecules originating from both the blastocyst and the uterus [57, 66]. These signaling molecules include the heparin-binding epidermal growth factor -like growth factor (HB-EGF) which has particular importance in embryo–uterine interactions and when deficient it limits pregnancy success in mice [6]. Das et al. [67] demonstrated that 6 hr prior to the blastocyst attachment, *HB-EGF* is expressed in the luminal epithelium and activates ErbB1/4 receptors located on the blastocyst; this endows the blastocyst with implantation competency. In the current study, the expression of *HB-EGF* was significantly upregulated by melatonin and consistent with the change of its receptor, *ErbB1*, in the blastocyst (Fig. 5). This finding suggests that melatonin acts on both the blastocyst and the uterus and promotes blastocyst activation in vivo.

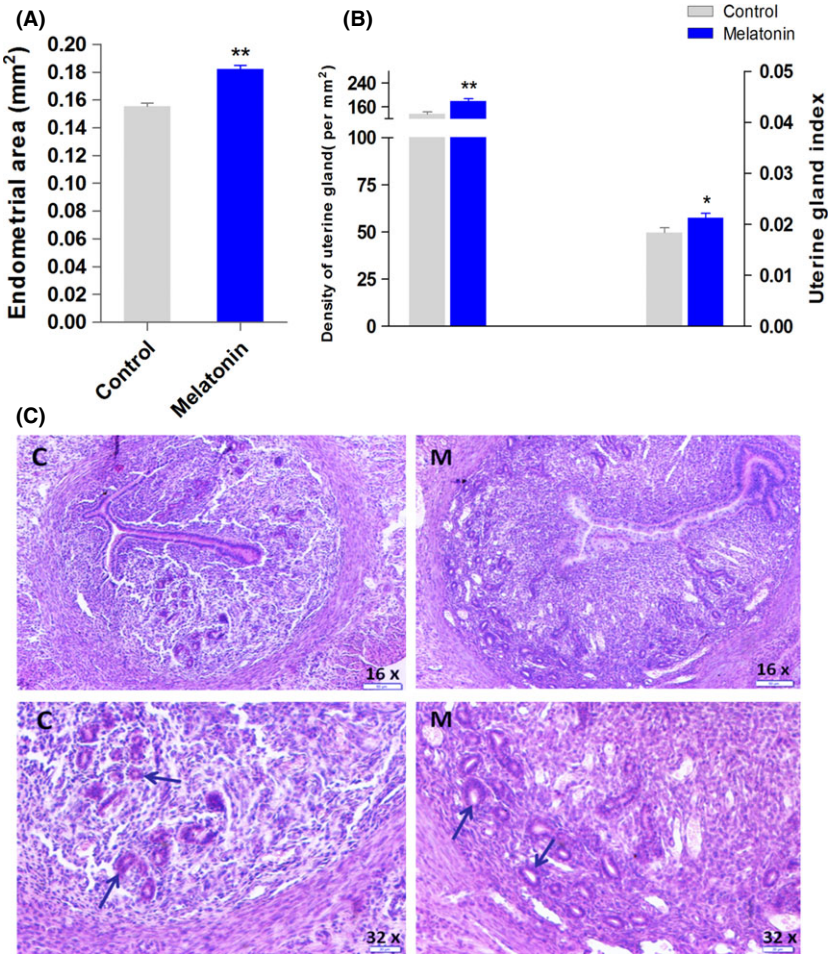

Fig. 3. Effect of melatonin on endometrium and uterine gland development. (A) The area of endometrium; (B) the density of uterine glands and uterine gland index, respectively; (C) a representative *H&E* section (C: control; M: melatonin). n = 10. *Represents significant differences, $P < 0.05$, **represents significant differences, $P < 0.01$.

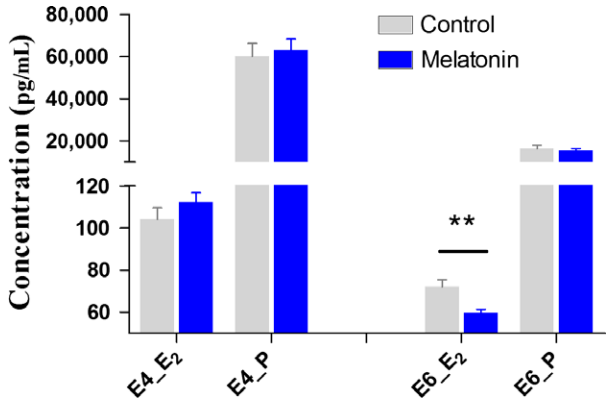

Fig. 4. Effect of melatonin on serum steroid hormone levels. A total of 113 mice were used for this experiment. **Represents significant differences, $P < 0.01$.

E2 and P are superior regulators of reproduction, and their functions are mediated by nuclear receptors *ERα* or *PRA*. Female mice that lack *ERα* or *PRA* are unable to support implantation due to defects in uterine physiology. P can promote endometrial luminal epithelial differentiation, stromal cell proliferation, and decidualization, which are mediated by PRA. $PRA^{-/-}$ mice manifest a dysfunctional uterine stroma and are unable to support embryo invasion and decidualization [68, 69]. Previous studies have proven that melatonin is involved in regulating E_2 and P in mammals [70, 71]. We detected an effect of melatonin on both the estrogen receptor-α (*ERα*) and progesterone receptor A (*PRA*); *PRA* expression was significantly upregulated in melatonin-treated mice (Fig. 5B). This action of melatonin has also been verified in the oviduct and ovary [71, 72]. Although there was no obvious change in serum P level (Fig. 4), its receptor *PRA* was upregulated by melatonin. Thus, we believe that melatonin can strengthen the function of P and *PRA* may be involved in mediating the beneficial effect of melatonin on endometrial development. It is noteworthy that the expression of *ERα* was not influenced by melatonin.

We also evaluated *p53*, a crucial gene involved in implantation on D4 through the regulation of LIF [8], which was significantly upregulated by melatonin treatment (Fig. 5B). Other important genes, such as cyclooxygenase1 (*Cox1*), Indian hedgehog (*Ihh*), homeobox A11 (*Hoax11*), and cannabinoid receptors (*CB1*), were not influence by melatonin (Fig. 5B).

Some studies demonstrated that melatonin benefits reproductive physiology mainly as a free radical scavenger and upregulating the expression of antioxidant or apoptosis-related genes [48]. In the current study, the

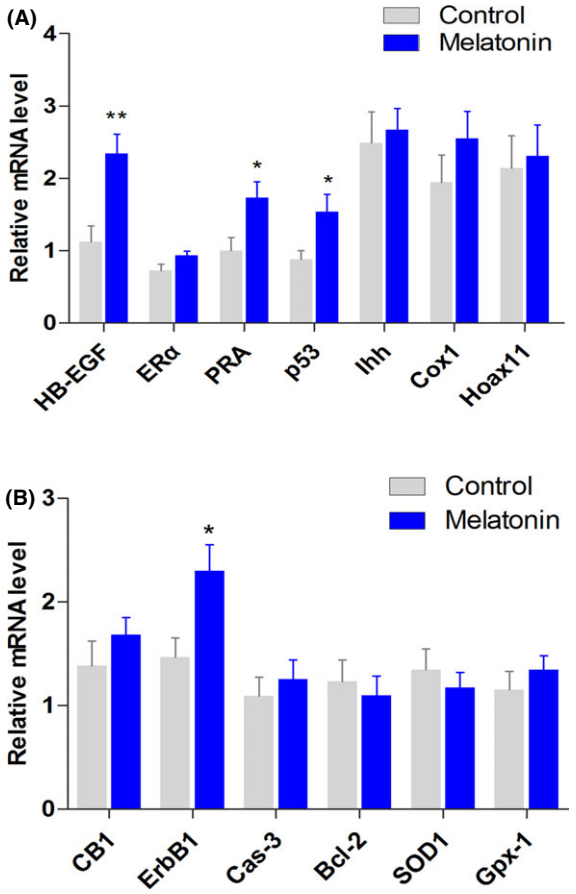

Fig. 5. Effects of melatonin on the implantation-related, apoptosis-associated, and anti-oxidative gene expression. (A) Genes in uterus, (B) genes in blastocyst. At least six repeats in each group. *Represents significant differences, $P < 0.05$, **represents significant differences, $P < 0.01$.

expression of *SOD1*, *Gpx-1* and *Caspase3*, *BCL-2* remained unchanged after melatonin treatment (Fig. 5A). We speculate that, under normal physiological conditions, carefully adjusted changes could keep oxidation and antioxidation in balance [73]; thus, antioxidant properties of melatonin are not involved in these events.

In conclusion, the rate-limiting enzyme of melatonin, *AANAT,* gradually increases during early gestation in mice, which suggests that the early pregnant uterus can synthesize melatonin. Moreover, the membrane receptor *MT2* is only expressed at D2, which hints that melatonin may participate in regulating uterine development or embryo implantation. The subsequent study using intraperitoneal melatonin injections confirmed that melatonin significantly improved the implantation number and litter size, possibly through enhancement of endometrium and uterine gland development by upregulating *PRA*, improving blastocyst activation and uterine receptivity by strengthening *ErbB1*, *HB-EGF,* and *p53* expression. The data obtained using a mouse model may provide valuable information for improving the implantation rate of assisted reproduction in women.

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