Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Melatonin improves endometrial receptivity of adenomyosis mice via the NF-κB signaling pathway

Kun-Ming Li (likunming@51mch.com)
Tongji University
Xiao-Hong Guan
Tongji University
Dan Liu
Tongji University
Hong Zhou
Tongji University
Chao-Qun Dai
Tongji University
Tao Wang
Tongji University
Yuan Fang
Tongji University
Yan-Ping Jia
Tongji University

Research Article

Keywords: adenomyosis, melatonin, endometrial development, NF-κB signaling pathway

Posted Date: April 20th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1539861/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

Background: Adenomyosis is a benign gynecological disorder but has detrimental effect on female fertility of childbearing age. Melatonin is a classic antioxidant and free radical scavenger to protect against tissue damage, and exerts important roles in reproductive systems. The effects of melatonin on endometrial development in adenomyosis remain unclear. This study aimed to explore the function of melatonin on endometrial development in adenomyosis mice and its possible mechanisms.

Methods: Inducing an adenomyosis mouse model by oral administration of tamoxifen. 0, 10, 20, 30, 50 mg/kg body weight melatonin were then respectively injected to investigate the effect of melatonin on implantation rates. Quantitative real time polymerase chain reaction (qRT-PCR), western blotting and immunohistochemistry were used to determine the expression of endometrial receptivity markers in endometrium during implantation window. Endometrial mRNA expressions of implantation-associated oxidative stress-correlated and apoptosis-related genes were analyzed by qRT-PCR. Western blotting was used to explore the mechanism of protective effect of melatonin on endometrial development.

Results: 30mg/kg melatonin injection significantly improved the number of implantation sites in an adenomyosis model mice. Adenomyosis adversely effected the development of mouse uterine development and impaired endometrial receptivity. Melatonin administration ameliorated hyper-inflammation state of the endometrium, improved antioxidant capacity and depressed apoptosis of endometrial cells induced by adenomyosis to reduce reproductive damage by suppressing the NF-κB signaling pathway.

Conclusion: Melatonin treatment ameliorates impaired endometrial development and endometrial receptivity of adenomyosis mice by improving the microenvironment of endometrium *via* NF-κB signaling pathway. The current study suggests the potential efficacy of melatonin based adenomyosis therapy.

Background

Adenomyosis, characterized by the presence of heterotopic endometrial glands and stroma in the myometrium, is extremely common in women of reproductive age, with an estimated prevalence of 38–64%[1]. Though it is a benign clinically condition but demonstrates a progressive and recurrent nature[2]. Adenomyosis also shows mysterious characteristics with adverse implications on women fertility and associated with lifelong infertility, which is identified in 27–79% of infertile women [3]. It negatively affects pregnancy and the live birth rate, as well as increases the risk of miscarriage. Compared with controls, adenomyosis patients had a 43% reduction in the odds ratio (OR) for clinical pregnancy [4]. Two recent meta-analysis[5, 6] concluded that adenomyosis is associated with a 30% decrease in the likelihood of pregnancy, with clinical pregnancy rates significantly reduced in in vitro fertilization/intracytoplasmic sperm injection as compared with those in control subjects (40.5% vs. 49.8%). Although the exact mechanisms by which adenomyosis may limit fertility remain elusive, alterations in endometrial receptivity have been suggested as one of the most important causes [7, 8].

Abnormal endometrial molecular expressions in adenomyosis have been thought to impair implantation and early embryo development. Inflammation processes such as interleukin-1β(IL-1b), tumor necrosis factor-α(TNF-α)[9, 10] and natural killer cells, macrophages[11] and a spectrum of cytoines[12] taking place within the eutopic endometrium induce cellular and biochemical alterations. Molecular markers associated with endometrial receptivity, homeobox A10/11 (Hox-A10 /11) gene and leukemia inhibitory factor (LIF) expression decreased in both mice models[13] and in the secretory phase endometrium of women with adenomyosis[14], whose dysregulation decrease down stream factors influencing endometrial receptivity by repressing target genes β3-integrin[15].

Melatonin (N-acetyl-5-methoxytryptamine) is a circadian hormone produced primarily by the pineal gland and acts as an anti-inflammatory agent, a powerful free radical scavenger, antioxidant, potential immune-regulator, and regulator of the circadian rhythm[16]. It is capable of suppressing the production of pro-inflammatory cytokines, including TNF-α and IL-1β, mainly by blocking nuclear factor kappaB(NF-κB) signaling[17]. Evidence suggests that melatonin and the reproductive system are interrelated under both physiological and pathological conditions[18]. Moreover, melatonin can optimize maternal, placental and fetal physiology[19], which is involved in the maintenance of uterine homeostasis through regulation of numerous pathways associated with uterine receptivity and gestation, and improves uterine physiological processes, such as decidualization and implantation[20]. Additionally, melatonin receptors (MR1A and MR1B) are localized in the glandular epithelial cells of the endometrium[21].Melatonin and its receptor(MT1)-mediate the anti-proliferative effects in the rat uterus[22], with other animal study showing that exogenous melatonin treatment can improve mouse embryo implantation and litter size [23]. These findings implicate melatonin may be useful for the infertility of adenomyosis. Consequently, a better understanding of the effects of melatonin on adenomyosis is needed.

Considering that the abnormal environment of endometrium in adenomyosis and the role of melatonin in female reproduction, we conducted a series of experiments to determine whether melatonin influences embryo implantation and endometrial receptivity in adenomyotic mice and further investigated its mechanism of action. To the best of our knowledge, this is the first study to evaluate the function relationship between melatonin and adenomyosis.

Materials And Methods

Animal Model

Female neonatal CD-1 mice (1.8-2.0g; 13 pregnant mice were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were orally dosed on days 2 through 5 after birth(birth day was set as day 1) with 2.7 μmol/kg tamoxifen (Shanghai Fudan Forward Science & Technology Co., Ltd, Shanghai, China) suspended in a peanut oil/lecithin/condensed milk mixture (2:0.2:3,v/v) at a dose volume of 5 μL/g body weight [13]. All mice were housed under controlled conditions of temperature (22–26°C) and light (12-/12-h light/dark cycle) with *ad libitum* access to food and water. After 75 days, tamoxifen-treated mice were randomly divided into five groups: Group I, adenomyosis model group; Group

II, melatonin 5 mg/kg body weight (Me 10); Group III, melatonin 10 mg/kg body weight (Me 20); Group IV, melatonin 15 mg/kg body weight (Me 30); and Group V, melatonin 25 mg/kg body weight (Me 50). Melatonin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in a small volume (0.02 mL) of absolute ethanol and diluted in 0.9%NaCl to a final concentration of 1 mg/ml. Mice from Groups II, III, IV, and V were intraperitoneally (*i.p.*) injected with melatonin every 12h at various doses (5, 10, 15 and 25 mg/kg body weight) for 4 weeks. Mice in the control group and adenomyosis model group received vehicle treatment of melatonin (NaCl + Ethanol). During days 110-125, all mice were mated with male mice from 19:00 to 07:00 and then checked for the presence of a vaginal plug at 08:00-9:00 the following day. The vaginal plug day was defined as gestation day 0.5. The animals were killed by cervical dislocation at day 8.5 for implantation site count. The optimal concentration of melatonin was determined by the number of implantation sites and used for the subsequent study. In a second experiment, another group of female mice received the same treatments described, after which the animals were euthanized at gestation day 4.5 (implantation window) of pregnancy, the uterus were removed for morphometric and endometrial receptivity analyses, and the oviducts were collected for blastocyst retrieval. Flow chart of the experiments can be seen in supplementFigure. All animal experiments were approved by the Ethics committee of the Tong-ji University (No. TJBG01321101) and complied with international guidelines for the care and use of laboratory animals.

Uterine Section and Morphometric Analysis

Uterine tissues from the experimental mice were harvested at night on day 4.5. Approximately 4/5 fractions of the uterus were used for collecting endometrial tissues, immediately dropped into liquid nitrogen, and stored at -80℃ for subsequent gene and protein analyses. Another 1/5 fraction of the uterine tissues were formalin-fixed and paraffin-embedded and cut into routine 5-μm-thick sections using a microtome for hematoxylin and eosin (H&E) staining and immunohistochemical(IHC) analysis. Images were observed under a Motic EasyScanner (Motic China Group Co., Ltd., Xiamen, China) and photographed. Six sections from each uterus were analyzed, and morphometric parameters were measured using Image Pro-Plus software (v.6.0; Media Cybernetics, Silver Spring, MD, USA). The interface between the myometrial and endometrial layers (P1) and the inner luminal surface area (P2) were traced, and the latter was subtracted from the former as the endometrial area (EA). The endometrial thickness index (ETI) was used to determine the thickness of endometrium, which was calculated using the formula [24, 25]:

$$ETI = 2EA/(P1 + P2)$$

Two investigators independently completed the measurement on different occasions.

RNA Extraction and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After the measurement of RNA concentration and quality, RNA was reverse transcribed to cDNA using PrimeScript RT reagent Kit (TaKaRa Bio, Shiga, Japan) according to manufacturer's instructions. The primers were designed and purchased from Sangon Biotechnology (Shanghai,China). qRT-PCR was performed using the TB Green Premix Ex Taq kit (TaKaRa Bio) and the Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. We detected expression levels of *glyceraldehyde 3-phosphate dehydrogenase (Gapdh)*, *Lif*, *integrin-β3, Hoxa10*, *Hoxa11*, *estrogen receptor α(Era), progesterone receptor α(Pra),P53,glutathione Peroxidase1(Gpx1), super Oxide Dismutase(Sod1), B-cell lymphoma-2(Bcl-2),* and *Bcl-2-associated X protein(Bax).* PCR conditions included 10μL TB Green, 0.4μL Dye II, 0.8μL forward and reverse primers, 2μL template, and ddH2O to a total volume of 20μL. The procedure was as follows: 95°C for 30s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s, a melting curve from 60 to 95°C with incremental increases of 0.5°C every 5 s. *GAPDH* was used an the standard control, and primer sequences are listed in Table1. Relative quantification of gene expression was performed using the $2^{-\triangle\triangle CT}$ method.

Western blot

Approximately 20mg of mouse endometrium was ground (Cebo Biotechnology, Shanghai, China) and lysed using radioimmunoprecipitation assay lysis buffer (Beyotime Biotechnology, Jiangsu, China) with a protease inhibitor cocktail (Thermo Fisher Scientific) on ice for 30 min. Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of total protein (30μg/lane) were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (EMD Millipore, Burlington, MA, USA) according to standard procedures. The membranes were then blocked using 5% non-fat milk (Sangon Biotech) or 5% bovine albumin (Sangon Biotech) in Tris-buffered saline with 0.05% Tween-20 detergent for 1.5 h on a shaker then incubated with primary antibody against GAPDH, Lif, Integrin-β3, Hoxa10, Hoxa11, p44/42 mitogen-activated protein kinase[MAPK; extracellular signal-regulated kinase (ERK) 1/2], phosphorylated(p)-p44/42MAPK(ERK1/2)(Thr202/Tyr204), p-p38 MAPK(Thr180/Tyr182), p38 MAPK, TNF-α, IL-1β, IκB-α, p-NF-κB p65(ser536), and NF-κB p65 at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies at room temperature for 1.5 h. Images were obtained using a chemiluminescent imaging system (Tanon Science & Technology Co., Ltd., Shanghai, China), with GAPDH was used as the internal control. Antibody information is presented in Table 2. The intensities of the protein bands were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

IHC Analysis

Formalin-fixed, paraffin-embedded uteri were subjected to routine 5μm thickness sectioning for IHC analysis. All IHC experiments were performed using a standard protocol. Briefly, sections were deparaffinized by incubating with xylene for 30 min and rehydrated in an ethanol series, followed by antigen retrieval and endogenous peroxidase blockage. The sections were then incubated with respective primary antibodies, including anti-α-smooth muscle actin (α-SMA), -Lif, - Integrin-β3, -Hoxa10 and -Hoxa11, overnight at 4°C in a humidified chamber, followed by incubation with the corresponding secondary antibodies. Phosphate-buffered saline was used as the negative control instead of the primary antibody. Antibody information is presented in Table 2. Image Pro-Plus software (Iv.6.0; Media Cybernetics) was used to evaluate the reactivity of the endometrial glands and luminal surface epithelium of the uterus (average positively-stained area percentage) using two independent analyzers.

Statistics analysis

All experiments were carried out at least three times, data represent the as mean ± standard deviation (SD). Statistical analyses were conducted using one-way analysis of variance, followed by Turkey's or Bonferroni *post hoc* analysis for multiple comparisons. All analyses were performed using GraphPad Prime software (GraphPad Software, LaJolla, CA, USA) and SPSS (v.18.0; SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

Results

Artificial induction of adenomyosis mouse model

A total of 96 mice were used for the first experiment. H&E pathologic andα-SMA staining analyses were used to verify the effectiveness of the mouse model(n=8). In control mice, the circular muscle layer was well developed to form an intact ring structure and without endometrial tissues (Fig.1.A, D), ectopic endometrial glands and/or stromal cells in the myometrium of all tamoxifen-induced mice (Fig.1.B,C and E,F) were visualized, indicating that neonatal mice treated with tamoxifen was a successful method of modeling adenomyosis. Further, the size of endometrial gland was smaller in melatonin injection mice than that in model mice (Fig.1.B vs C, E vs F).

Melatonin improves implantation sites and endometrium development of adenomyosis mice

To clarify whether melatonin can improve pregnancy outcomes in adenomyosis model mice, we administered melatonin via *i.p.* injection to the model mice (n=12). The results showed that adenomyosis adversely influenced pregnancy outcome, with the number of implantation sites more significantly decreased in adenomyosis model mice than in normal mice (5.0 ± 2.10 vs 13.3 ± 2.38, p 0.0001). By contrast, 30mg/kg melatonin treatment significantly increased the number of implantation sites relative

to that observed in untreated adenomyosis model mice (9.0 ± 0.63 vs 5.0 ± 2.10, p=0.002) Fig. 2.A , B. Therefore, we determined 30mg/kg melatonin as the optimal concentration for subsequent studies.

To clarify whether the treatments affected ovulation, blastocysts were flushed from uteri at day 4.5(n=12), revealing no differences between three groups (Fig. 2.C). To elucidate the mechanism, the endometrium development was analyzed(n=6). Adenomyosis negatively effects endometrial development in mice; however, melatonin treatment reversed the development of the damaged endometrium, with both the EA and ETI showing improvements in melatonin-treated mice relative to untreated adenomyosis model mice (p 0.01), although these measurements remained lower than those in control animals (p 0.05 and p 0.0001, respectively). (Fig.2.D-F).

Melatonin reversed impaired endometrial receptivity

To explore the mechanism of the beneficial effect of melatonin on implantation, we measured gene and protein expression of implantation-related markers in the endometrium of uteri during the implantation window(n=6). We observed altered endometrial gene expression in the adenomyosis mouse model, with adenomyotic endometrium showing significant decreases in *Lif, Integrin-β3 , Hoxa10,* and *Hoxa11* mRNA levels relative to those in control mice (p 0.0001, respectively). Their levels were significantly upregulated after melatonin treatment (p 0.05, respectively) (Fig. 3.A). Additionally, changes in protein levels of these markers corresponded to those of the mRNA levels (Fig.3.B, C).

Furthermore, IHC staining revealed that Lif and Integrin-β3 were mainly located in the cytoplasm of endometrial luminal epithelial cells and glandular epithelial cells, whereas Hoxa10 and Hoxa11 were expressed in the nuclei of endometrial luminal epithelial cells and glandular epithelial cells (Fig.3.D). Quantitative analysis of IHC data revealed that levels of Lif, Integrin-β3, Hoxa10, and Hoxa11 were significantly lower in adenomyosis mice than those in control group (p 0.001, respectively), whereas melatonin treatment significantly recovered these levels (p 0.05, respectively) (Fig.3.E). These results indicated that melatonin alleviated damaged endometrial receptivity in the adenomyosis mouse model.

Melatonin alleviates aberrant endometrial metabolism

To further explore the potential mechanisms of the beneficial effects of melatonin on endometrial receptivity, we extracted mRNA from eutopic endometrium of mice and determined changes in the expression of implantation-related (*Era, Pra*, and *P53*), oxidative-stress-related (*Sod1* and *Gpx1*), and apoptosis-related (*Bcl-2* and *Bax*) genes by qRT-PCR (n=6). The results showed significantly lower *Era* expression in the adenomyosis model group relative to that in the control and melatonin-treated groups (p< 0.05) (Fig.4.A), whereas *Pra* expression remained unchanged (p 0.05) (Fig.4.B), and *P53* expression was significantly upregulated in the melatonin-treated group relative to that in the adenomyosis model group (p< 0.001)(Fig.4.C). Oxidative stress is considered an important factor affecting the female reproductive system, and melatonin is reportedly involved in redox homeostasis[26]. In the present study,

we found that adenomyosis altered the expression of oxidative-stress-related genes, whereas melatonin treatment ameliorated oxidative stress by significantly upregulated the expression of *Sod1* ($p< 0.05$) (Fig.4.E) but not *Gpx1*, the expression of which was higher in adenomyosis model mice than in the control group ($p< 0.01$) (Fig.4.D). Excessive oxidative stress often leads to apoptosis. As expected, expression of the anti-apoptotic gene *Bcl-2* was lower and that the ratio of *Bax/Bcl-2* was higher in adenomyosis model mice than the other two groups ($p< 0.05$), whereas melatonin treatment markedly upregulated *Bcl-2* ($p< 0.05$) and downregulated the ratio of *Bax/Bcl-2* expression relative to that in the untreated model group ($p< 0.05$) (Fig.4.F, H). These results indicated that melatonin exerted a protective effect against oxidative stress induced by adenomyosis and reduced endometrial apoptosis to improve endometrial receptivity.

Melatonin reduces abnormal inflammatory responses in adenomyosis mice by suppressing NF-κB signaling

To investigate the molecular mechanisms of melatonin underlying the protective effects of melatonin on adenomyosis model mice, we first investigated changes in levels of the proinflammatory cytokines TNF-α and IL-1β in the endometrium of mice during the implantation window (n=6). Western blot analysis showed that adenomyosis model mice showed higher TNF-α and IL-1β levels relative to the control ($p< 0.0001$ and $p< 0.01$, respectively) (Fig.5.B, D). A previous study reported that melatonin exerts an anti-inflammatory effect during the treatment of diseases[27]. Therefore, we examined whether melatonin inhibits the release of proinflammatory cytokines in the endometrium of mice with adenomyosis, with the results confirming that melatonin significantly reduced TNF-α and IL-1β levels($p< 0.001$ and $p< 0.05$, respectively) (Fig.5.B, D). MAPK and NF-κB signaling pathways are involved in melatonin-regulated cell functions[17, 28]. In the present study, we determined the relative contributions of p38 MAPK, p-p38 MAPK, ERK, p-ERK□IκB-α□NF-κB and p-NF-κB in melatonin-mediated suppression of TNF-α and IL-1β production in endometrial tissue at day 4.5(n=6). However, western blot results revealed no differences in levels of p38 MAPK, p-p38 MAPK, ERK, or p-ERK among the three groups (Fig.5.A, C), suggesting that melatonin treatment reduces the secretion of proinflammatory cytokines in adenomyosis, although not via the MAPK and ERK signaling pathways.

NF-κB, a critical transcriptional factor involved in inflammation and inactive when bound to its specific inhibitor IκB, is a common downstream target of the phosphoinositide 3-kinase/AKT and ERK pathways[29]. During NF-κB activation, IκB kinase induces IκB degradation, resulting in the activation and translocation of NF-κB to the nucleus, where it is reportedly involved in regulation the expression of genes encoding proinflammatory cytokines and their receptors[30]. NF-κB activation is also enhanced by TNF-α stimulation, and according to the feed-forward relationship between proinflammatory factors and NF-κB activation, it is likely that activated NF-κB may result in increased production of proinflammatory chemokines [10]. Therefore, we determined whether melatonin treatment attenuated TNF-α and IL-1β production via the NF-κB pathway. The results showed reduced levels of IκB-α in adenomyosis model group relative to that in the control group($p< 0.0001$), and that melatonin treatment restored its expression ($p< 0.05$) (Fig.5.B, D). Additionally, adenomyosis induced higher levels of p-NF-κB p65 and the p-NF-κB

p65/ NF-κB p65 ratio relative to those observed in control mice(p< 0.0001 and p< 0.05, respectively), whereas melatonin administration significantly reduced both of these(p< 0.0001 and p< 0.01, respectively) (Fig.5.B, D). These data suggested that NF-κB may play a pivotal role in the development of adenomyosis by promoting inflammation, apoptosis and impairing endometrial development in mice (Fig.6). Furthermore, the results indicated that melatonin might reduce TNF-α and IL-1β production in the endometrium of adenomyosis mice, thereby suppressing the NF-κB signaling.

Discussion

Adenomyosis is one the common disease in women of reproductive age, manifestsing a variable degree of symptoms while causing reproductive failure in a high percentage of patients [4]. The adjuvant measures to improve pregnancy outcomes have been extensively explored, but the results is not satisfactory. Melatonin produced mainly by the pineal gland during the darkness is a multifunctional agent, with increasing evidence identifying melatonin-specific functions in female reproduction [31]. Additionally, melatonin receptors have been identified in ovarian [32] and uterine [21, 23] tissues. However, information regarding the function of melatonin in adenomyosis is limited. In the present study, our results showed that adenomyosis had a detrimental effect on uterine receptivity in mice, and that melatonin therapy significantly reversed the impaired endometrial receptivity by reducing inflammatory reaction, oxidative stress, and apoptosis by suppressing NF-κB signaling pathway.

The endometrium is the primary target organ for embryo implantation, and the development state of endometrium is directly related to embryo survival and implantation, as well as the establishment and maintenance of gestation [33]. During early gestation, melatonin injection could improve embryo implantation and litter size in a mouse model[23]. Additionally, pinealectomy significantly reduced the success of rat embryo implantation, and exogenous melatonin supplementation remodeled endometrial morphology and improved embryo implantation[24]. In the present study, we examined the roles of melatonin in uterine development of adenomyosis model mice by injecting melatonin into the peritoneal cavity for 4 weeks. The results showed that 30mg/kg melatonin increased the number of implantation sites, which is consistent with the previous report [23]. Moreover, we observed no obvious change in the number of recovered blastocysts between the three groups.

The correlation between endometrial thickness (Eth) and the ability of embryos to implant has been analyzed, and there is a general consensus that a higher Eth results in a better chance of implantation [34]. Additionally, Eth is closely related to EA. In the present study, we found that endometrium morphology developed much more poorly in adenomyosis model mice than in control mice. We observed a “thin” endometrium, which is likely the morphologic expression of defective vascularization during the invasive phase; however, this morphology improved following melatonin administration but had no effect on ovulation, which indirectly indicated that melatonin replacement may effectively restore normal physiological function to the endometrium in adenomyosis model mice. To better clarify the effect of melatonin on endometrium development in adenomyosis model mice, we identified downregulated expression of endometrial receptivity markers (Fig. 4) in the endometrium, which agreed with findings

from previous report [13].Dysregulation of endometrial receptivity markers in the endometrium during the implantation window suggested that adenomyosis correlated with impaired implantation; however, these markers were significantly up-regulated after melatonin supplement. These data provided further evidence of the protective effect of melatonin on endometrial development in adenomyosis model mice.

In females, estradiol (E_2) and progesterone(P) play crucial roles in multiple reproductive systems, and their functions are mediated by the nuclear receptors ERA and PRA. During pre-implantation, a small surge of E_2 is critical for inducing mouse uterine receptivity. Blastocyst can locally synthesize and secrete E_2 to initiate implantation [35], and that knockout of nuclear estrogen and progesterone receptors leads to complete infertility [36, 37]. In our study, we observed reduced *Era* expression on day 4.5 in adenomyosis model mice, and that exogenous melatonin supplementation actively upregulated this expression to sensitize the uterus, although no change was observed in *Pra* expression. The same trend was observed in *P53* expression, a gene critical for implantation. This might be because low local estrogen level in the endometrium of adenomyosis model mice dysregulated matrix remodeling during the implantation window, leading to poor endometrial development and further damaging embryo implantation.

Excessive free radical environments can damage fertilized eggs and interfere with embryo development. Melatonin benefits reproductive physiology mainly as a free radical scavenger and upregulates the expression of antioxidant- or apoptosis-related genes[38]. When reactive oxygen species (ROS) reach a maximum resistance threshold, tissue damage or even cell death can occur. Here, we found dramatic decrease in *Sod1* mRNA levels in adenomyosis model mice, but that these were upregulated following melatonin treatment. Notably, we did not observed melatonin-mediated changes in *Gpx-1* expression. This may be explained by the necessity for high levels of GPX1 in order to remove excessive peroxide ions in the endometrium of adenomyosis mice. Melatonin may act as direct ROS scavenger and regulate endogenous antioxidant enzymes, such as SOD, to decrease levels of oxidative stress. Excessive oxidative stress often leads to apoptosis, which is an important regulator of eutopic endometrial function. Previous studies demonstrated that increased cellular proliferation and decreased apoptosis of endometrial cells facilitate ectopic survival and implantation of adenomyosis [39]. Our data showed that adenomyosis altered the expression of apoptosis-associated genes, induced a variable degree of apoptosis, hindered endometrial development, and impaired endometrial receptivity, whereas melatonin administration improved the expression of anti-apoptosis-associated genes and decreased the apoptosis rate in the endometrium of adenomyosis model mice. These results showed that melatonin supplementation might effectively mitigate oxidative stress-induced apoptosis by maintaining redox homeostasis, thereby enabling the endometrium to promote embryo implantation and rescue the adverse outcomes caused by adenomyosis.

Adenomyosis is a hyperinflammatory disease and inflammatory factors are involved in cell adhesion, proliferation, apoptosis and invasion. Abnormal inflammatory responses and ROS are toxic to embryos, ultimately impairing endometrial receptivity and thereby damaging pregnancy outcomes. In the present study, we identified elevated TNF-α and IL-1β levels in the endometrium of adenomyosis model mice

along with decreased *Sod1* expression. We speculated that the presence of an increased inflammatory reaction and oxidative stress in the mouse model damaged endometrium development.

In addition to regulating circadian rhythms, melatonin also exerts antioxidant, anti-inflammatory, and immunomodulatory activities in mammals, with its anti-inflammatory effect involving several signaling pathways, such as those related to MAPK, NF-κB, and MMPs [17, 40]. These wide-spectrum, anti-inflammatory activities eventually lead to blockade of the NF-κB signaling pathway. Endometrial alterations are partically responsible for infertility and suboptimal uterine receptivity, and the abnormal endometrial milieu may contribute to adverse pregnancy outcomes. The pathogenesis of adenomyosis subfertility, which has not been fully elucidated, was the focus of present study. NF-κB is an important nuclear transcription factor, and NF-κB-mediated signaling plays an important role in inflammation [41]. Melatonin can maintain the inner blood-retinal barrier by inhibition the p38/thioredoxin-interacting protein/NF-κB pathway in diabetic retinopathy [28]. NF-κB activation occurs following signal-induced degradation of IκB-α, resulting in NF-κB translocation to the nucleus, where it regulates the expression of specific genes. In the present study, we identified lower IκB-α levels and higher p-NF-κB p65 levels in adenomyosis model mice, with melatonin supplementation reversing this effect. This result suggested that melatonin might inhibit inflammation and improve endometrial receptivity via NF-κB pathway.

Looking back on our study, there are some limitations. First, the experiment about offsprings was not performed, and further experiments are required to conduct. Second, to better elucidate the role of melatonin, we will knock out melatonin receptors in adenomyosis model to determine the exact mechanism of melatonin.

Conclusions

In summary, we identified impaired endometrial receptivity in an adenomyosis mouse model, as well as reversal of this harmful effect by administration of antioxidant melatonin. Specifically, melatonin injection inhibited the hyper-inflammation state in the mouse model by improving the expression levels of endometrial receptivity markers, which was accompanied by an increase in embryo implantation via inhibition of NF-κB signaling. These results demonstrated the protective effect of physiological melatonin on adenomyosis, especially the positive melatonin-specific effects on endometrial receptivity, and highlight its potential as an auxiliary therapeutic candidate for the treatment of adenomyosis.

Abbreviations

Me: melatonin ;TNF-α: tumor necrosis factor-α;IL-1β: interleukin-1β; qRT-PCR: Quantitative real time polymerase chain reaction; H&E: hematoxylin and eosin staining; IHC: immunohistochemical analysis; α-SMA:anti-α-smooth muscle actin;Gapdh: glyceraldehyde 3-phosphate dehydrogenase; Lif: leukemia inhibitory factor; Hoxa10/11: homeobox A10/11; Era: estrogen receptor α; Pra: progesterone receptor α; Gpx1: glutathione Peroxidase1; Sod1: super oxide dismutase; Bcl-2: B-cell lymphoma-2; Bax: Bcl-2-associated X protein; NF-κB: nuclear factor kappaB; ERK 1/2: p44/42 mitogen-activated protein

kinase[MAPK; extracellular signal-regulated kinase], p-ERK1/2(Thr202/Tyr204):phosphorylated(p)-p44/42MAPK; Eth :endometrial thickness.

Declarations

Acknowledgments

We thank Dr. Qizhi He for his technical assistance.

We would like to thank Editage (www.editage.com) for English language editing.

Author Contributions

XHG, YPJ and KML designed the study. XHG performed the majority of the experiments. XHG and DL wrote the main manuscript. DL, HZ and YF contributed to the regents and materials. XHG and CQD conducted specimens. XHG, TW and YPJ analyzed the data. All authors reviewed the manuscript and agree to submit.

Funding

This work was supported by the Natural Science Foundation of Shanghai, China (21ZR1450700).

Availability of data and materials

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Ethics approval and consent to participate

The study was approved by the Ethics committee of the Tong-ji University, Shanghai, China (No. TJBG01321101).

Consent for publication

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Competing interests

The authors declare that they have no competing interests.

References

1. Naftalin J, Hoo W, Pateman K, Mavrelos D, Foo X, Jurkovic D: **Is adenomyosis associated with menorrhagia?** *Hum Reprod* 2014, **29**(3):473-479.

2. Chapron C, Vannuccini S, Santulli P, Abrão MS, Carmona F, Fraser IS, Gordts S, Guo S-W, Just P-A, Noël J-C *et al*: **Diagnosing adenomyosis: an integrated clinical and imaging approach**. *Human reproduction update* 2020, **26**(3):392-411.
3. Kunz G, Beil D, Huppert P, Noe M, Kissler S, Leyendecker G: **Adenomyosis in endometriosis–prevalence and impact on fertility. Evidence from magnetic resonance imaging**. *Human reproduction (Oxford, England)* 2005, **20**(8):2309-2316.
4. Horton J, Sterrenburg M, Lane S, Maheshwari A, Li TC, Cheong Y: **Reproductive, obstetric, and perinatal outcomes of women with adenomyosis and endometriosis: a systematic review and meta-analysis**. *Hum Reprod Update* 2019, **25**(5):592-632.
5. Vercellini P, Consonni D, Dridi D, Bracco B, Frattaruolo MP, Somigliana E: **Uterine adenomyosis and in vitro fertilization outcome: a systematic review and meta-analysis**. *Hum Reprod* 2014, **29**(5):964-977.
6. Younes G, Tulandi T: **Effects of adenomyosis on in vitro fertilization treatment outcomes: a meta-analysis**. *Fertil Steril* 2017, **108**(3).
7. Munro MG: **Uterine polyps, adenomyosis, leiomyomas, and endometrial receptivity**. *Fertility and sterility* 2019, **111**(4):629-640.
8. Karel C, Michal J, Radovan P, Pavel V, Jana Ž, Jan V, Tomáš C: **Adenomyosis - its possible effect on endometrial function and receptivity**. *Ceska Gynekol* 2021, **86**(3):205-209.
9. Yen C-F, Liao S-K, Huang SJ, Tabak S, Arcuri F, Lee C-L, Arici A, Petraglia F, Wang H-S, Kayisli UA: **Decreased Endometrial Expression of Leukemia Inhibitory Factor Receptor Disrupts the STAT3 Signaling in Adenomyosis During the Implantation Window**. *Reprod Sci* 2017, **24**(8):1176-1186.
10. Li B, Chen M, Liu X, Guo S-W: **Constitutive and tumor necrosis factor-α-induced activation of nuclear factor-κB in adenomyosis and its inhibition by andrographolide**. *Fertil Steril* 2013, **100**(2):568-577.
11. Carrarelli P, Yen C-F, Funghi L, Arcuri F, Tosti C, Bifulco G, Luddi A, Lee C-L, Petraglia F: **Expression of Inflammatory and Neurogenic Mediators in Adenomyosis**. *Reprod Sci* 2017, **24**(3):369-375.
12. Zhihong N, Yun F, Pinggui Z, Sulian Z, Zhang A: **Cytokine Profiling in the Eutopic Endometrium of Adenomyosis During the Implantation Window After Ovarian Stimulation**. *Reprod Sci* 2016, **23**(1):124-133.
13. Guo S, Li Z, Yan L, Sun Y, Feng Y: **GnRH agonist improves pregnancy outcome in mice with induced adenomyosis by restoring endometrial receptivity**. *Drug Des Devel Ther* 2018, **12**:1621-1631.
14. Fischer CP, Kayisili U, Taylor HS: **HOXA10 expression is decreased in endometrium of women with adenomyosis**. *Fertil Steril* 2011, **95**(3):1133-1136.
15. Du H, Taylor HS: **The Role of Hox Genes in Female Reproductive Tract Development, Adult Function, and Fertility**. *Cold Spring Harb Perspect Med* 2015, **6**(1):a023002.
16. Yong W, Ma H, Na M, Gao T, Zhang Y, Hao L, Yu H, Yang H, Deng X: **Roles of melatonin in the field of reproductive medicine**. *Biomed Pharmacother* 2021, **144**:112001.
17. Huang C-C, Chiou C-H, Liu S-C, Hu S-L, Su C-M, Tsai C-H, Tang C-H: **Melatonin attenuates TNF-α and IL-1β expression in synovial fibroblasts and diminishes cartilage degradation: Implications for the**

treatment of rheumatoid arthritis. *J Pineal Res* 2019, **66**(3):e12560.

18. Yang H-L, Zhou W-J, Gu C-J, Meng Y-H, Shao J, Li D-J, Li M-Q: **Pleiotropic roles of melatonin in endometriosis, recurrent spontaneous abortion, and polycystic ovary syndrome**. *American journal of reproductive immunology (New York, NY : 1989)* 2018, **80**(1):e12839.
19. Reiter RJ, Tan DX, Korkmaz A, Rosales-Corral SA: **Melatonin and stable circadian rhythms optimize maternal, placental and fetal physiology**. *Human reproduction update* 2014, **20**(2):293-307.
20. Chuffa LGdA, Lupi LA, Cucielo MS, Silveira HS, Reiter RJ, Seiva FRF: **Melatonin Promotes Uterine and Placental Health: Potential Molecular Mechanisms**. *International journal of molecular sciences* 2019, **21**(1).
21. Mosher AA, Tsoulis MW, Lim J, Tan C, Agarwal SK, Leyland NA, Foster WG: **Melatonin activity and receptor expression in endometrial tissue and endometriosis**. *Human reproduction (Oxford, England)* 2019, **34**(7):1215-1224.
22. Zhao H, Pang SF, Poon AMS: **mt(1) Receptor-mediated antiproliferative effects of melatonin on the rat uterine antimesometrial stromal cells**. *Mol Reprod Dev* 2002, **61**(2):192-199.
23. He C, Wang J, Li Y, Zhu K, Xu Z, Song Y, Song Y, Liu G: **Melatonin-related genes expressed in the mouse uterus during early gestation promote embryo implantation**. *J Pineal Res* 2015, **58**(3):300-309.
24. Dair EL, Simoes RS, Simões MJ, Romeu LRG, Oliveira-Filho RM, Haidar MA, Baracat EC, Soares JM: **Effects of melatonin on the endometrial morphology and embryo implantation in rats**. *Fertility and sterility* 2008, **89**(5 Suppl):1299-1305.
25. Rossi AGZ, Soares JM, Motta ELA, Simões MJ, Oliveira-Filho RM, Haidar MA, Rodrigues de Lima G, Baracat EC: **Metoclopramide-induced hyperprolactinemia affects mouse endometrial morphology**. *Gynecol Obstet Invest* 2002, **54**(4):185-190.
26. Yanar K, Simsek B, Çakatay U: **Integration of Melatonin Related Redox Homeostasis, Aging, and Circadian Rhythm**. *Rejuvenation Res* 2019, **22**(5):409-419.
27. Chitimus DM, Popescu MR, Voiculescu SE, Panaitescu AM, Pavel B, Zagrean L, Zagrean A-M: **Melatonin's Impact on Antioxidative and Anti-Inflammatory Reprogramming in Homeostasis and Disease**. *Biomolecules* 2020, **10**(9).
28. Tang L, Zhang C, Yang Q, Xie H, Liu D, Tian H, Lu L, Xu J-Y, Li W, Xu G *et al*: **Melatonin maintains inner blood-retinal barrier via inhibition of p38/TXNIP/NF-κB pathway in diabetic retinopathy**. *Journal of cellular physiology* 2021, **236**(8):5848-5864.
29. Hoesel B, Schmid JA: **The complexity of NF-κB signaling in inflammation and cancer**. *Mol Cancer* 2013, **12**:86.
30. Cao S, Zhang X, Edwards JP, Mosser DM: **NF-kappaB1 (p50) homodimers differentially regulate pro- and anti-inflammatory cytokines in macrophages**. *J Biol Chem* 2006, **281**(36):26041-26050.
31. Carlomagno G, Minini M, Tilotta M, Unfer V: **From Implantation to Birth: Insight into Molecular Melatonin Functions**. *International journal of molecular sciences* 2018, **19**(9).

32. Zhang L, Zhang Z, Wang J, Lv D, Zhu T, Wang F, Tian X, Yao Y, Ji P, Liu G: **Melatonin regulates the activities of ovary and delays the fertility decline in female animals via MT1/AMPK pathway**. *J Pineal Res* 2019, **66**(3):e12550.
33. Kelleher AM, DeMayo FJ, Spencer TE: **Uterine Glands: Developmental Biology and Functional Roles in Pregnancy**. *Endocr Rev* 2019, **40**(5):1424-1445.
34. Richter KS, Bugge KR, Bromer JG, Levy MJ: **Relationship between endometrial thickness and embryo implantation, based on 1,294 cycles of in vitro fertilization with transfer of two blastocyst-stage embryos**. *Fertility and sterility* 2007, **87**(1):53-59.
35. Zhang S, Lin H, Kong S, Wang S, Wang H, Wang H, Armant DR: **Physiological and molecular determinants of embryo implantation**. *Mol Aspects Med* 2013, **34**(5):939-980.
36. Kayo D, Zempo B, Tomihara S, Oka Y, Kanda S: **Gene knockout analysis reveals essentiality of estrogen receptor β1 (Esr2a) for female reproduction in medaka**. *Sci Rep* 2019, **9**(1):8868.
37. Fang X, Wu L, Yang L, Song L, Cai J, Luo F, Wei J, Zhou L, Wang D: **Nuclear progestin receptor (Pgr) knockouts resulted in subfertility in male tilapia (Oreochromis niloticus)**. *J Steroid Biochem Mol Biol* 2018, **182**:62-71.
38. Tamura H, Takasaki A, Taketani T, Tanabe M, Lee L, Tamura I, Maekawa R, Aasada H, Yamagata Y, Sugino N: **Melatonin and female reproduction**. *J Obstet Gynaecol Res* 2014, **40**(1).
39. Li J, Yanyan M, Mu L, Chen X, Zheng W: **The expression of Bcl-2 in adenomyosis and its effect on proliferation, migration, and apoptosis of endometrial stromal cells**. *Pathol Res Pract* 2019, **215**(8):152477.
40. Qin W, Li J, Zhu R, Gao S, Fan J, Xia M, Zhao RC, Zhang J: **Melatonin protects blood-brain barrier integrity and permeability by inhibiting matrix metalloproteinase-9 via the NOTCH3/NF-κB pathway**. *Aging (Albany NY)* 2019, **11**(23):11391-11415.
41. Yin Y, Chen F, Wang W, Wang H, Zhang X: **Resolvin D1 inhibits inflammatory response in STZ-induced diabetic retinopathy rats: Possible involvement of NLRP3 inflammasome and NF-κB signaling pathway**. *Mol Vis* 2017, **23**:242-250.

Tables

Table 1. Primers for qRT-PCR

Genes	Primer sequence(5'-3')	Product size(bp)	Tm(℃)
Gapdh	Forward:AGGTCGGTGTGAACGGATTTG	123	60.88
	Reverse:TGTAGACCATGTAGTTGAGGTCA		58.59
Lif	Forward:GCTGTATCGGATGGTCGCATA	156	60.07
	Reverse:CACAGACGGCAAAGCACATT		59.69
Itgb3	Forward:GGCGTTGTTGTTGGAGAGTC	138	59.41
	Reverse:CTTCAGGTTACATCGGGGTGA		59.45
Hoxa10	Forward:GGCAGTTCCAAAGGCGAAAAT	86	60
	Reverse:GTCTGGTGCTTCGTGTAAGGG		60.94
Hoxa11	Forward:ATAGCACGGTGGGCAGGAACG	96	65.06
	Reverse:AGTCGGAGGAAGCGAGGTTTT		61.71
Era	Forward:TGTCCAGCAGTAACGAGAAAGG	94	60.29
	Reverse:TGGTAGCCAGAGGCATAGTCAT		60.69
Pra	Forward:CTCCGGGACCGAACAGAGT	128	60.98
	Reverse:GCGGGGACAACAACCCTTT		60.83
P53	Forward:TGAGGTTCGTGTTTGTGCCTGC	165	64.06
	Reverse:CCATCAAGTGGTTTTTTCTTTTGC		58.19
Gpx1	Forward:CCACCGTGTATGCCTTCTCC	105	60.46
	Reverse:AGAGAGACGCGACATTCTCAAT		59.57
Sod1	Forward:CACTCTCAGGAGAGCATTCCA	110	59.17
	Reverse:CCCAGCATTTCCAGTCTTTG		56.98
Bcl-2	Forward:ACCTGTGGTCCATCTGACCCTC	163	63.42
	Reverse:CCAGTTCACCCCATCCCTGA		61.21
Bax	Forward:AGACAGGGGCCTTTTTGCTAC	137	60.55
	Reverse:AATTCGCCGGAGACACTCG		60.15

Table 2. The information for the primary antibodies.

Antibody	Catalog number	Application(conc.)	Host	Target species	Vendor
anti-GAPDH	#5174	WB(1:1000)	Rabbit	Mouse/Human	Cell Signaling Technology
anti-LIF	DF13730	WB(1:1000) IHC(1:50)	Rabbit	Mouse/Human	Affinity
anti-ITGB3	AF6086	WB(1:1000) IHC(1:50)	Rabbit	Mouse/Human	Affinity
anti-HOXA10	#58891	WB(1:1000) IHC(1:50)	Rabbit	Mouse/Human	Cell Signaling Technology
anti-HOXA11	ab72591	WB(1:1000) IHC(1:50)	Rabbit	Mouse/Human	Abcam
anti-p44/42 MAPK (ERK1/2)	#9102	WB(1:1000)	Rabbit	Mouse/Human	Cell Signaling Technology
anti-p-p44/42 MAPK (ERK1/2)	#4370	WB(1:2000)	Rabbit	Mouse/Human	Cell Signaling Technology
anti-p38 MAPK	T55600	WB(1:1500)	Rabbit	Mouse/Human	Abmart
anti-p-p38 MAPK	T40076	WB(1:1500)	Rabbit	Mouse/Human	Abmart
anti-TNF-α	AF7014	WB(1:500)	Rabbit	Mouse/Human	Affinity
anti-IL-1β	AF5103	WB(1:1000)	Rabbit	Mouse/Human	Affinity
anti-IκB-α	TA7776	WB(1:1500)	Rabbit	Mouse/Human	Abmart
anti-p-NF-κB p65	TA2006	WB(1:1500)	Rabbit	Mouse/Human	Abmart
anti-NF-κB p65	TA5006	WB(1:1500)	Rabbit	Mouse/Human	Abmart
anti-α-SMA	AF1032	IHC(1:50)	Rabbit	Mouse/Human	Affinity

Figures

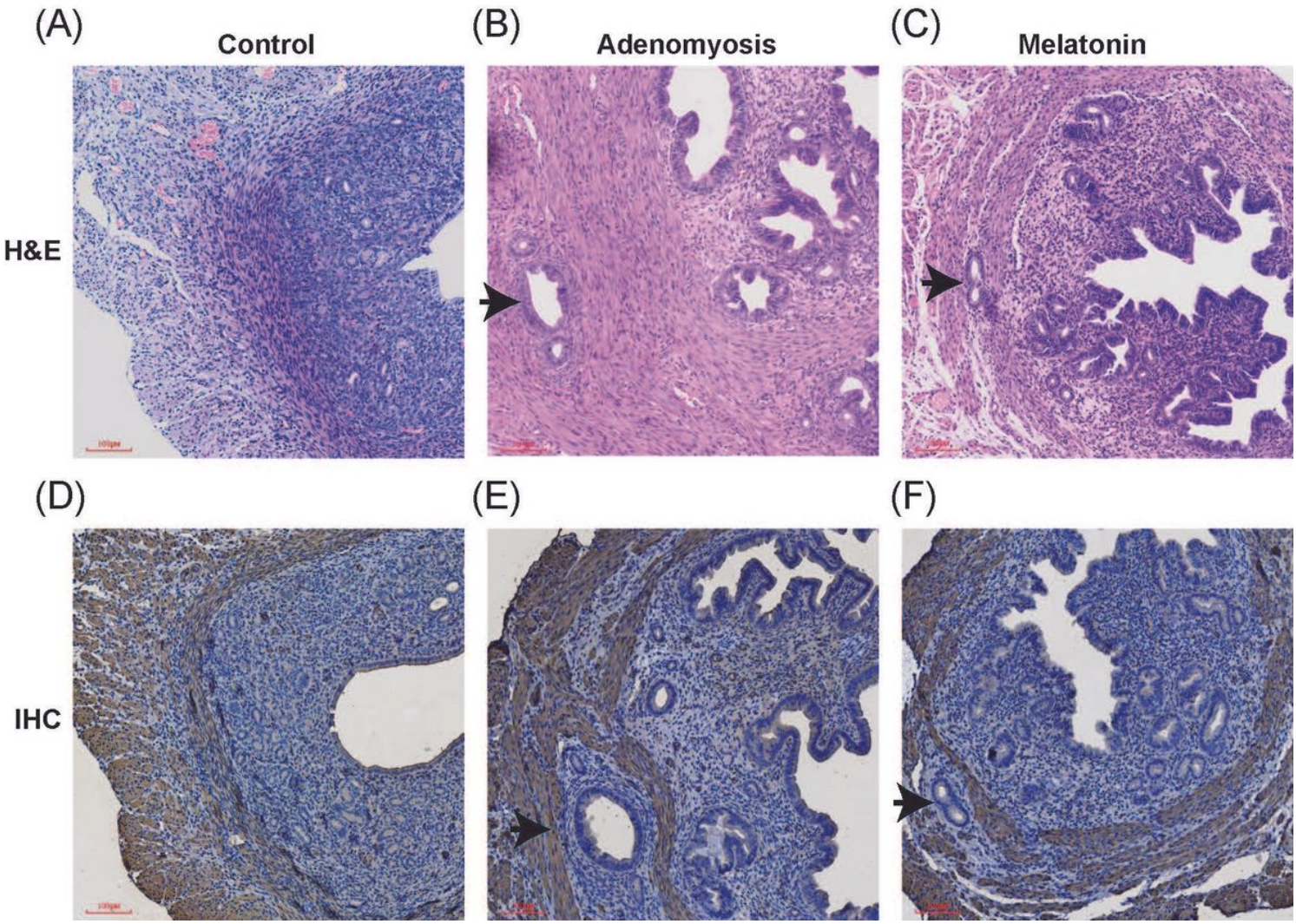

Figure 1

Light microscopy of uteri. A,**D** Representative images from the control mice, **B**,**E** the adenomyosis model group, **C**, **F** the melatonin-treatment group according to H&E staining and α-smooth muscle actin staining (magnification: 100×). The arrows depict the ectopic endometrial glands.

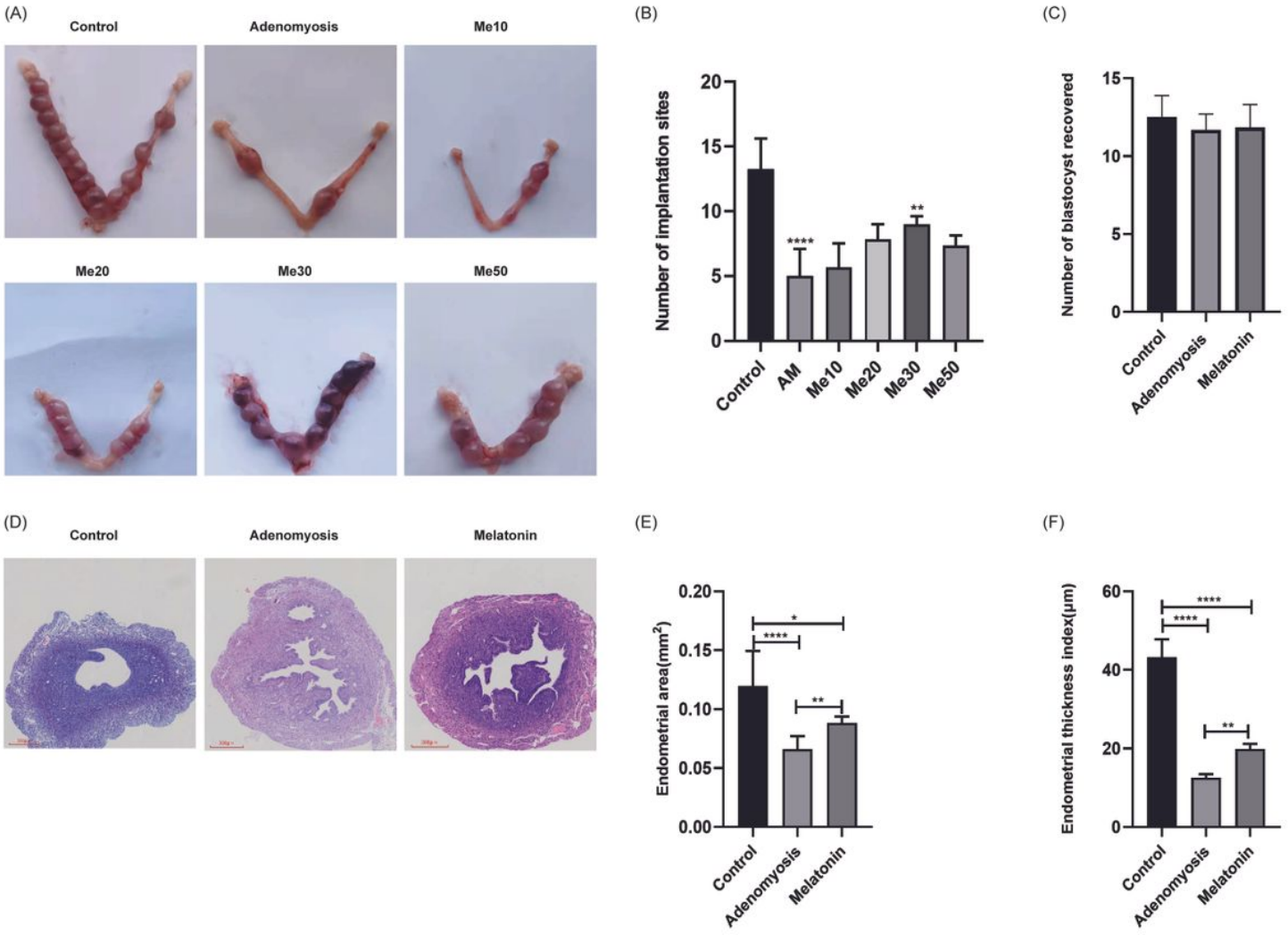

Figure 2

Effect of melatonin on the number of implantation sites and endometrial development. A , B Number of implantation sites. **C N**umber of recovered blastocysts. **D** Representative H&E sections showing endometrial development (magnification: 40×). **E** The EA and **F** ETI (μm). (*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$).

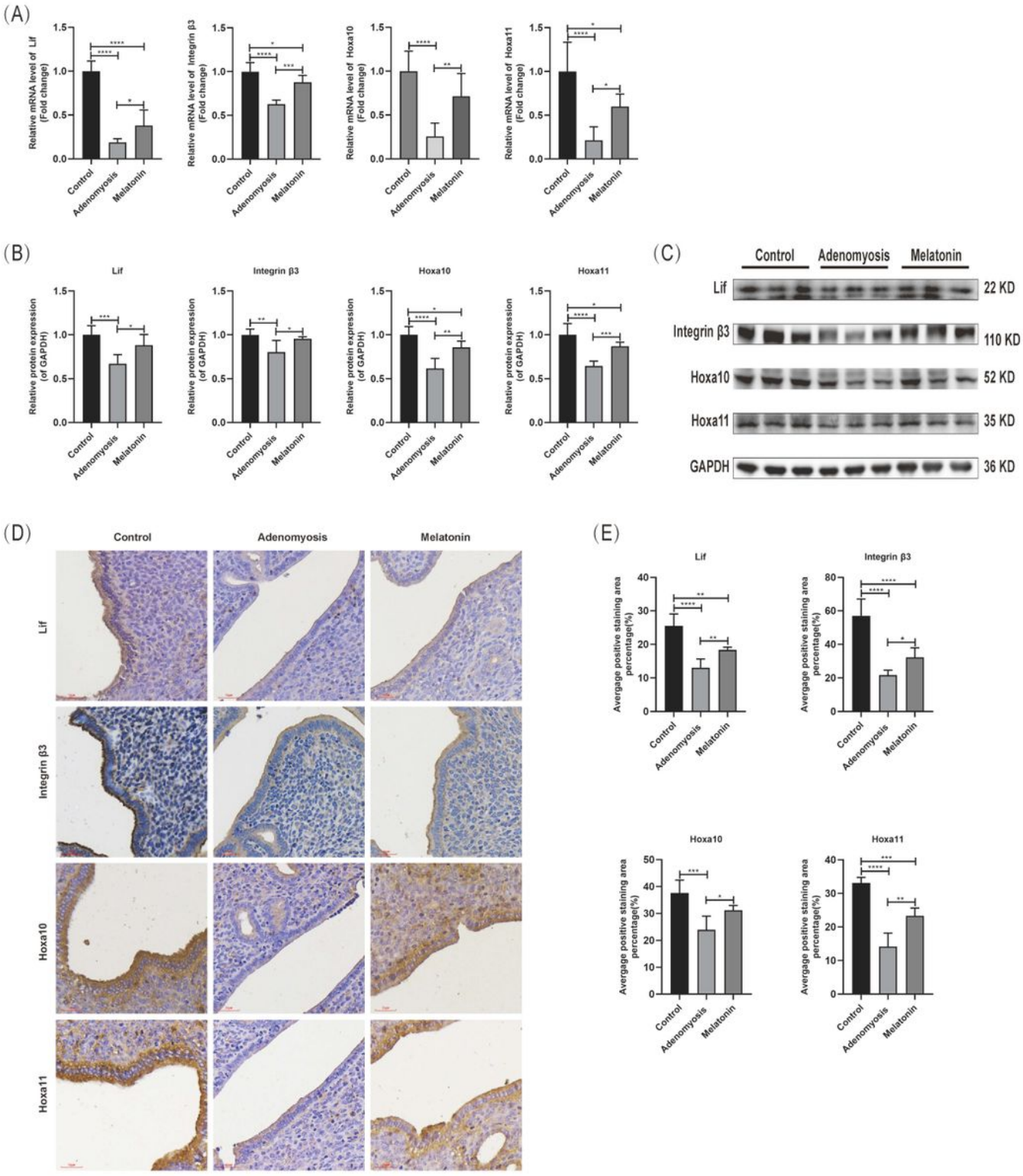

Figure 3

The effects of melatonin on endometrial receptivity during the implantation window. A mRNA levels of endometrial receptivity markers(*Lif*, *integrin-β3, Hoxa10* and *Hoxa11*) were evaluated by qRT-PCR in endometrium during implantation window from the control, adenomyosis model and melatonin treated mice. **B** Protein levels of endometrial receptivity markers of Lif, integrin-β3, Hoxa10 and Hoxa11 in control, adenomyosis model and melatonin treated group were detected by western blotting, evaluated by Image

J software, and normalized against GAPDH. **C** A representative western blotting images of Lif, integrin-β3, Hoxa10 and Hoxa11. **D** Representative images of immunohistochemistry for Lif, integrin-β3, Hoxa10 and Hoxa11(magnification: ×400) and **E** their quantified expression values. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

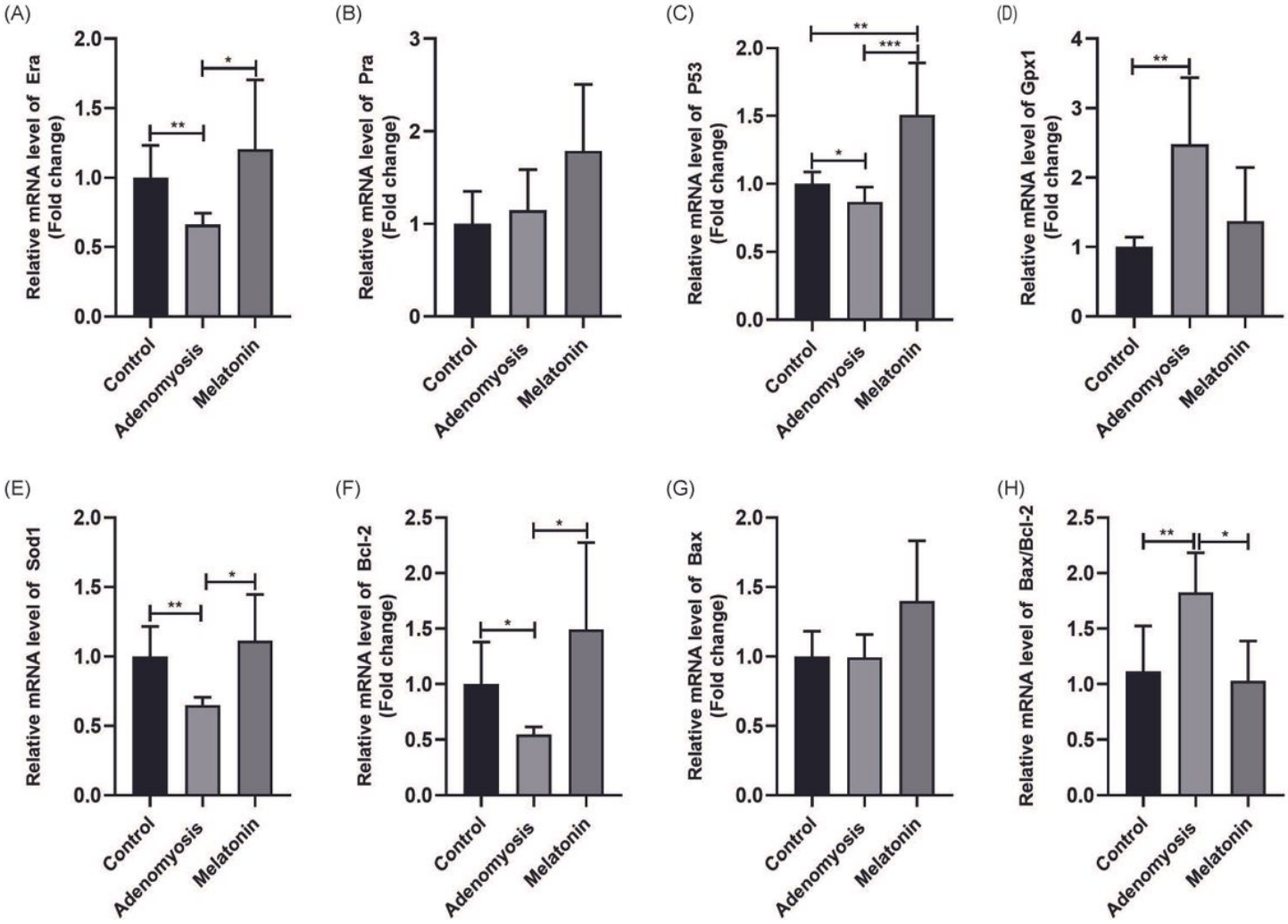

Figure 4

Effects of melatonin on the implantation-related, antioxidative-associated and apoptosis gene expression. A, B and C mRNA expression of implantation- related genes *Era, Pra*, and *P53,* **D** and **E** antioxidative-associated genes *Gpx* and *Sod1,* **F**, **G** and **H** apoptosis-correlated genes of *Bax, Bcl-2* and the ratio of *Bax/Bcl-2* in endometrium during implantation window from mice of the control, adenomyosis model and melatonin treated groups. (*$p < 0.05$; **$p < 0.01$).

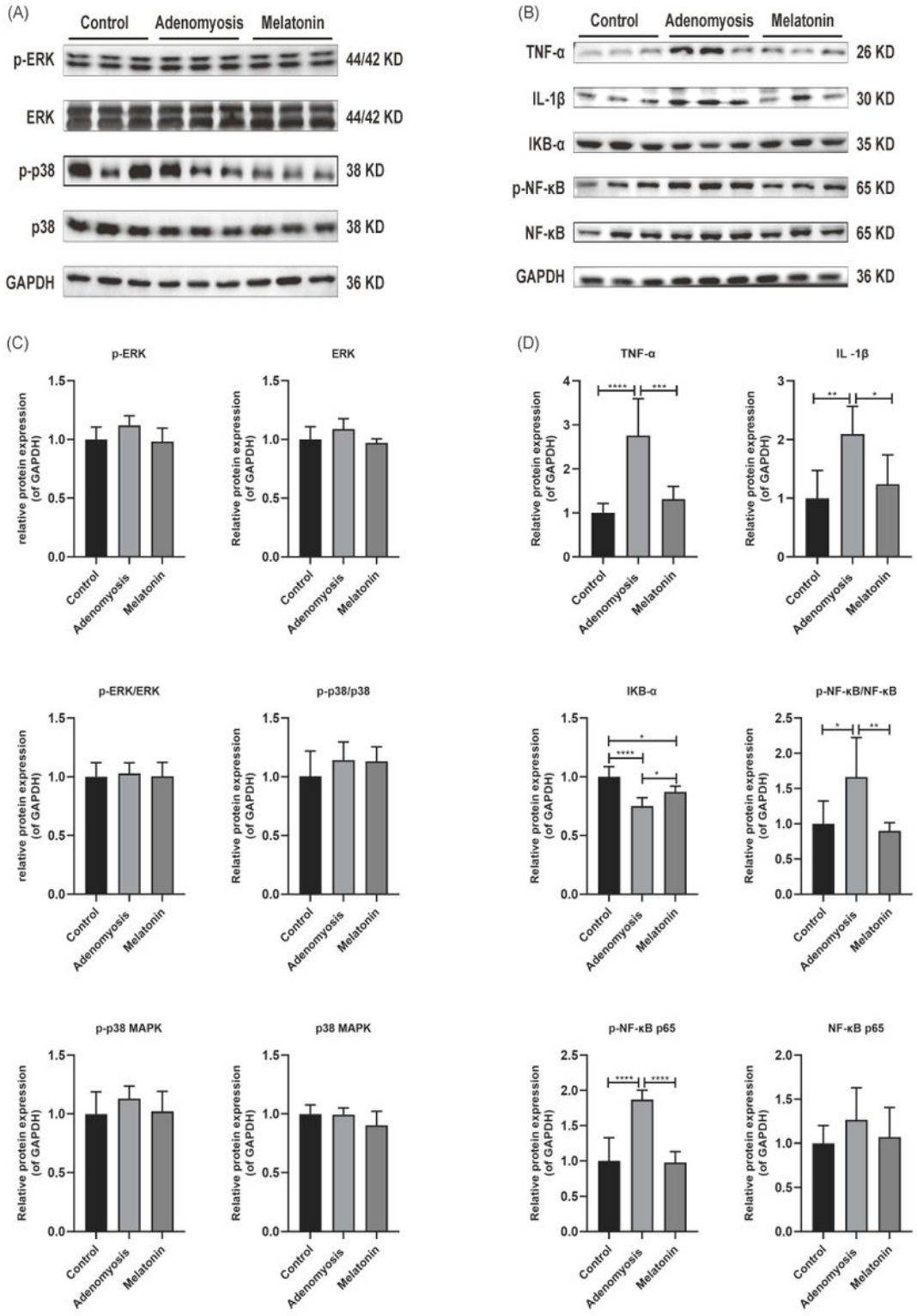

Figure 5

Melatonin inhibits the expression of inflammatory factors in adenomyosis model mice via the NF-κB signaling pathway. A The representative western blotting images of p-ERK, ERK, p-p38 and p38 in endometrium during implantation window from mice of the control, adenomyosis model and melatonin treated groups. **B** The representative western blotting images of TNF-α, IL-1β, IκB-α, p-NF-κB p65, and NF-κB p65 of each group. **C** Quantitative analysis of p-ERK, ERK and the p-ERK/ERK ratio, p-p38, p38 as well

as the p-p38/p38 ratio normalized against GAPDH. **D** Quantitative analysis of TNF-α, IL-1β, IκB-α, p-NF-κB p65, and NF-κB p65 levels, as well as the p-NF-κB/NF-κB ratio, normalized against GAPDH.(*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

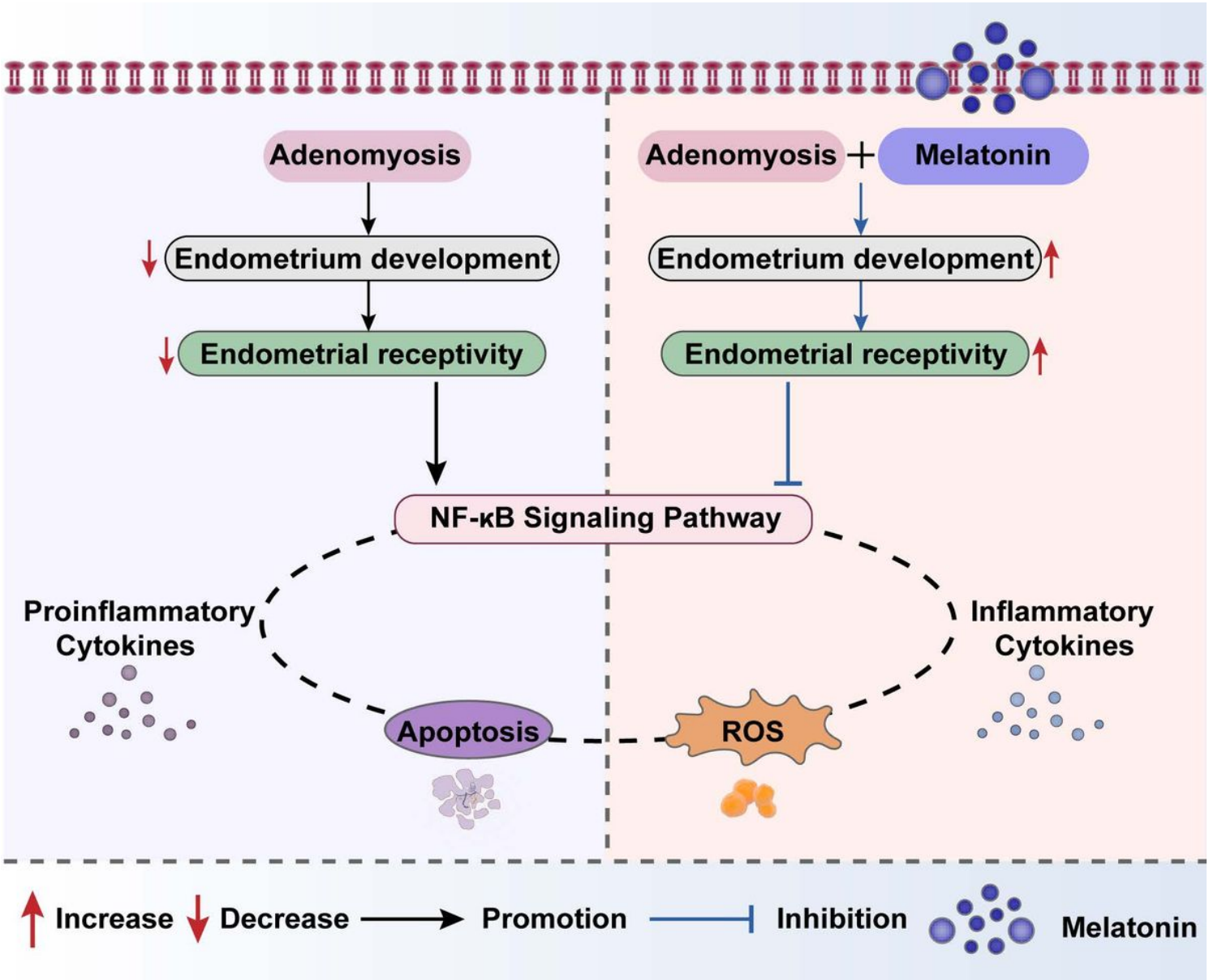

Figure 6

A schematic diagram of the regulatory mechanism of melatonin in adenomyosis model mice.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementFigrue.jpg