Melatonin Ameliorates The Toxicity Induced By Deoxynivalenol In Murine Ovary Granulosa Cells By Antioxidative And Anti-Inflammatory

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Research

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Abstract

Background: Deoxynivalenol (DON) is a common fusarium mycotoxin contaminant of food and feedstuff, posing a serious threat to human and animal reproductive system by inducing oxidative stress and inflammation. The antioxidant melatonin participates in immunoregulation, playing a crucial role in animal development. So, our present study aimed to explore DON-induced toxicity in murine ovary granulosa cells (GCs), and assess the potential protective effect of melatonin.

Results: Melatonin inhibits DON-induced oxidative stress in murine ovary GCs by decreased the generation of intracellular reactive oxygen species, ameliorates DON-induced mitochondrial dysfunction. And inhibits the inflammatory response through inhibits reduce the activation of NF-κB and phosphorylation of ERK, JNK, and p38 MAPKs induced by DON. Moreover, melatonin significantly improved the down-regulation of reproductive hormone gene expression induced by DON contaminant. These results once again demonstrate the properties of melatonin: antioxidant and anti-inflammatory.

Conclusions: Melatonin have beneficial effects on mitigating the toxicity induced by deoxynivalenol in ovary Granulosa Cells. Offer a novel therapeutic strategy against mycotoxin contamination-induce diseases

Introduction

Deoxynivalenol (DON) is the main mycotoxin secreted by the *Fusarium* fungus, which frequently contaminates grain and feed [1, 2]. Generally, contamination with DON is accompanied by contamination with other mycotoxins, such as 3-acetyl-deoxynivalenol (3-acetyl-DON), 15-acetyl-deoxynivalenol (15-acetyl-DON), and DON-glucoside [3], which possess the same main functional groups as DON. A previous study demonstrated that even though extrusion cooking can effectively promote the degradation of DON, it cannot completely eliminate DON [4]. The high chemically stability of DON allows it to be easily absorbed via feed intake into the gastrointestinal tract [5], and then enter various other organs [6]. Consequently, DON exerts severe detrimental effects, causing a series of pathophysiological symptoms in humans and animals, including nausea, diarrhea, vomiting, leukocytosis, hemorrhage, and even death [7]. Therefore, DON contamination poses a serious threat to human health and animal husbandry. Animal experiments have shown that acute exposure to high doses of DON induces oxidative stress [8], leukocytosis, gastrointestinal bleeding, circulatory failure, and eventually death. Meanwhile, exposure to longterm medium dose food intake resulted in endocrine changes, which can cause body weight decrease, immune dysfunction, and can affect development and animal fertility [9]. In the reproductive system, DON inhibits the proliferation of ovarian granulosa cells (GCs) and endometrial cells [10, 11], and alters the synthesis of testosterone, progesterone, and estradiol in ovarian GCs [12, 13], which adversely affects oocyte maturation and embryo development [11, 14]. Moreover, the specialized structure of ovarian GCs with oocytes has a crucial effect on the development of follicles, oocytes [15, 16], subsequent zygote quality, and embryonic development. However, the specific mechanisms of the toxic effects of DON on murine ovary GCs remain unclear.

Melatonin (N-acetyl-5-methoxytryptamine) is a major hormone secreted by the pineal gland, which is involved in biological rhythm regulation and seasonal reproductive activity in mammals [17]. Recent research reported that melatonin can be produced by many other tissues, including the gastrointestinal tract [18], immune cells [19], and reproductive system cells [20, 21]. Furthermore, it has been widely used in a variety of products to improve the quality of human sleep. Meanwhile, melatonin is also widely recognized as a natural antioxidant and free radical scavenger [11], and has been shown recently to have pleiotropic effects [17, 22]. Melatonin and its metabolites have been reported to activate a variety of antioxidant enzymes, and are involved in antioxidative stress and the inhibition of cell apoptosis [23]. In the reproductive system, accumulating research has proven that melatonin plays an important role via regulation of antioxidative stress, especially the steroidogenesis capacity in the development of ovarian GCs [16], oocytes [11], pronuclear embryo, embryo implantation, and litter size [24]. Melatonin supplementation in pig ovary GCs *in vitro* contributed to the synthesis of estradiol, helped to regulate the mRNA expression of reproductive hormone-related genes [16], and upregulated the mRNA expression of oocyte maturation associated genes, such as *GDF9* (growth differentiation factor 9) and *DNMT1A* (DNA methyltransferase 1) [25]. Moreover, Melatonin protected porcine oocytes from heat stress [23] and Di-2-ethylhexyl phthalate (MEHP) (plasticizer) [26] exposure-induced meiosis defects via its antioxidant properties. In addition, melatonin could effectively eliminate free radicals in oocytes during ovulation [21]. However, it is unclear whether melatonin could rescue the toxic effects of DON exposure on mouse ovary GCs.

In the present study, we aimed to investigate the toxic effects and mechanisms of DON exposure on murine ovary GCs, and to explore whether melatonin administration could protect against the detriments induced by DON.

Methods

Chemicals and reagents

DON (D0156; 5 mg) and Melatonin (M5250; 250 mg) were purchased form Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM)-F12 were obtained from Gibco BRL (Invitrogen Corporation, Carlsbad, CA, USA). Anti-BCL2 associated X, apoptosis regulator (Bax; ET1603-34), anti-BCL2 apoptosis regulator (Bcl-2; ET1603-11), anti-caspase 3 (ER30804), anti-cleaved caspase 3 (ET1608-64), anti-caspase 9 (ET1603-27), anti-follicle stimulating hormone receptor (FSHR; ER1909-08), and anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase (HRP; HA1031) antibodies were purchased form Hangzhou HuaAn Biotechnology (Hangzhou, China). Anti-androgen receptor (AR; ab273500), Anti-extracellular regulated kinase 1 (ERK1; ab109282), anti-phospho-ERK (ab201015), anti-p65 (ab32536), anti-phospho-p65 (ab76302), anti-p38 (ab170099), anti-phospho-P38 (ab178867), anti-IkappaBalpha (IκB) (ab32518), anti-phospho-IKB (ab133462), anti-JNK (ab179461), and anti-phospho-JNK (ab124956) antibodies were obtained from Abcam Ltd. (Cambridge, UK). Anti-heat shock protein 90 (HSP90; 60318) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 10494-1-AP) antibodies were obtained from Proteintech Ltd. (Proteintech, Rosemont, IL, USA).

Isolation, purification, and culture of ovarian GCs

Four-week-old Institute of Cancer Research (ICR) female mice were housed in an appropriate temperature-controlled room with a 12-hour light-dark cycle, and fed with a regular diet and water. Mice were injected with 6 IU pregnant mare serum gonadotrophin, Ningbo Hormone Products Co, Zhejiang, China), and then at 44–48 hours later, their ovaries were collected. Ovarian GCs were collected from the isolated ovaries and washed three times in serum-free DMEM/F12. The GCs were dispersed with DMEM/F12 supplemented with 10% FBS, and seeded at a density 5×10^4 cells/ml in 96-well and 6-well plates. The cells were incubated in 5% CO_2 at 37 °C for 24 hours before further treatment, and the culture medium was refreshed every 24 hours.

DON and melatonin treatment

DON was dissolved in ethanol and diluted in culture medium to a final concentration of 2 μM. Melatonin was dissolved in ethanol and diluted in culture medium to 2 μM DON-contaminated culture medium to final concentrations of 0.1, 1.0, 10, 100, and 1000 μM. The final concentration of the solvent was less than 0.01% in the culture medium. Cells treated with control (an equal volume of solvent), DON (2 μM), melatonin (1.0 μM) and DON (2 μM) + melatonin (1.0 μM) were analyzed in subsequent trials.

Assessment of cell viability

After the GCs grew to 70% confluency in 96-well plates, they were treated with DON (2 μM), Melatonin (0.1, 1.0, 10, 100, and 1000 μM), and DON (2 μM) + Melatonin (0.1, 1.0, 10, 100, and 1000 μM) for 24 hours. Cell viability was assessed using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Tokyo, Japan) according to the manufacturer's protocol. The optical density (OD) measurement was carried out at a wavelength of 450 nm on a Tecan Infinite 200 microplate reader (Sunrise, Tecan, Switzerland).

Analysis of apoptosis using flow cytometry

After the GCs grew to 70% confluency in 6-well plates, they were treated with DON (2 μM), Melatonin (1.0 μM), and DON (2 μM) + Melatonin (1.0 μM) for 24 hours, and the control group was cultured in medium with an equal volume of solvent. GCs were collected to checked apoptosis via Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection Kit (Solarbio, Beijing, China). Briefly, collected GCs were washed in ice cold phosphate-buffered saline (PBS), stained with Annexin V-FITC and PI in order, subjected to flow cytometry, and then analyzed using CytExpert 2.3 (Beckman Coulter, Brea, CA, USA), each measurement was performed three times using at least 10,000 cells.

Measurement of Reactive Oxygen Species (ROS) using flow cytometry

ROS in GCs were monitored using flow cytometry using a Reactive Oxygen Species Assay Kit (Solarbio). After treatment with DON (2 μM), Melatonin (1.0 μM) and DON (2 μM) + Melatonin (1.0 μM) for 24 hours, the cells were collected, washed in pre-cooled PBS twice, and then incubated with 10 μM Dichloro-dihydro-fluorescein diacetate (DCFH-DA) in culture medium at 37 ℃ for 30 min. The cells were collected,

washed in PBS three times, resuspended in PBS, and then the intracellular ROS were measured using a flow cytometer (Beckman Coulter).

Measurement of the Mitochondrial Membrane Potential ($\Delta\Psi m$)

The Mitochondrial Membrane Potential ($\Delta\Psi m$) was determined quantitatively using a JC-1 dye Detection Kit (Beyotime, Jiangsu, China) by flow cytometry. JC-1 dye is an ideal fluorescent probe to detect the $\Delta\Psi m$, and is suitable for cells, tissues, and purified mitochondria. When the $\Delta\Psi m$ was high, JC-1 accumulates in the mitochondrial matrix to from JC-1 aggregates, presenting as red fluorescence; however, when the $\Delta\Psi m$ is lower, JC-1 dye accumulates in the cytoplasm as monomers, presenting as green fluorescence. Thus, it is easy to detect a decrease in the $\Delta\Psi m$ by observing the transition from red fluorescence to green fluorescence of JC-1. Therefore, the ratio of the red to green fluorescence intensity, as detected using flow cytometry, represents the quantitative $\Delta\Psi m$ in each group. Briefly, after the GCs were treated with DON (2 μM), Melatonin (1.0 μM) and DON (2 μM) + Melatonin (1.0 μM) for 24 hours, they were collected and stained by JC-1 dye at 37 ℃ for 20 min. The cells were then washed three times and resuspended in 0.5 ml of per-cooled buffer solution, and the fluorescence was quantitatively determined using CytExpert 2.3 (Beckman Coulter).

Determination of the ATP content

The ATP content of GCs was determined using an Enhanced ATP Assay Kit (Beyotime). The treated GCs from the four groups were collected and counted using cell-count boards, and then diluted to the same number of cells (1×10^7) in each group. The ATP content was then determined following the manufacturer's instructions. Briefly, the collected cells were lysed with lysis buffer on ice for 20 mins, and then centrifuged at 12,000 × *g* for 5 min at 4 °C, and the supernatant was retained. Meanwhile, the ATP working solution was added at 100 μl/well), and kept at room temperature for 5 mins. Then, ATP standard solutions diluted at different concentration and the different supernatant samples were added to 96-well plate (20 μl/well), mixed with ATP working solution quickly, and subsequently measured using a BioTek Synergy 2 Multimode Microplate Reader (BioTek, Winooski, VT, USA). The ATP content was quantified according to the established standard curve.

RNA Extraction and quantitative real-time reverse transcription PCR (qRT-PCR)

The GCs were collected after treatment with DON (2 μM), Melatonin (1.0 μM) and DON (2 μM) + Melatonin (1.0 μM) for 24 hours in a 6-well plate. Then, total RNA was extracted using a YEASEN Total RNA Extraction Reagent (YEASEN, Shanghai, China), and the RNA quantity was measured using 260/280 UV spectrophotometry. Equal quantities of RNA were reverse transcribed into cDNA using an HiScript® Q RT SuperMix for qRT-PCR (+gDNA wiper) kit (Vazyme, Nanjing, China). Then, quantitative real-time PCR (qPCR) reactions were carried out using the cDNA as the template and the AceQ® qPCR SYBR® Green Master Mix (Vazyme) in an ABI StepONEPlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), using the following protocol: 95 ℃ for 5 mins; followed by 40 cycles of 95℃ for 10 seconds, 60℃

for 30 seconds. All the specific primers used for qPCR are listed in Table S1. The mouse *Gapdh* mRNA was used as an internal control. Relative gene expressive was calculated using the $2^{-\Delta\Delta Ct}$ method [27].

Western Blotting Analysis

The treated GCs were collected, washed with pre-cooled PBS, and then lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors for 10 min on ice, and centrifuged at 13 000 × *g* for 10 min at 4 °C. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Biosharp, Beijing, China). Equal amounts of protein (20 μg) were loaded in each well and separated on an 8% or 10% sodium dodecyl sulphate–polyacrylamide gel, and then transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were washed thrice with Tris-Buffered Saline Tween-20 (TBST) and then blocked with 5% nonfat dry milk in TBST at room temperature for 2 hours with gentle agitation. Subsequently, the blocked membrane were incubated with primary antibodies (diluted 1:1000 at 4 ℃ overnight. The membranes were washed thrice with TBST (10 minutes each), and then incubated with anti-rabbit IgG-HRP secondary antibody (1:5000) at room temperature for 1 hour with gentle agitation. The membranes were washed thrice with TBST (10 minutes each), and then incubated with Luminol/Enhancer Reagent (New Cell &Molecular Biotech, Suzhou, China) and exposed to the FluorChem FC3 system (Protein-Simple, San Jose, CA, USA). Finally, the relative integrated density of each protein band was digitized using the FluorChem FC3 system. GAPDH or HSP90 was selected as a control for equal protein loading.

Data analysis and statistics

The results are presented as means ± SD with at least three samples, or the mean ± SEMs using at least three biological replicates. Statistical analyzes were performed and the figures were generated using GraphPad Prism software (version 8.0, GraphPad Inc, San Diego, CA, USA). Statistical comparisons used an unpaired t-test. *P* value 0.05 was considered statistically significant.

Results

Effect of melatonin on DON-induced cell viability

The cell viability of ovarian GCs treated with DON, Melatonin, and DON+Melatonin for 24 hours was determined using the CCK-8 assay (Fig. 1). When ovary GCs were treated with melatonin separately at different concentration (0.1, 1.0, 10, 100 and 1000 μM), only melatonin of 1000 μM decreased cell viability significantly ($P < 0.05$) (Fig. 1a). We treated ovary GCs with DON (2 μM) and DON (2 μM) + melatonin (0.1, 1.0, 10, 100 and 1000 μM). The result showed that DON (2 μM) decreased cell viability significantly ($P < 0.01$), whereas co-treatment with low concentration of melatonin (0.1, 1.0, and 10 μM) attenuated the cytotoxicity of DON significantly (Fig. 1b). The cell confluency of the DON (2 μM) + melatonin (1.0 μM) group was significantly higher than that in the DON-treat group, and the cell

morphology also seemed normal (Fig. S1). These results revealed that an appropriate amount of melatonin could rescue the cytotoxicity induced by DON in ovarian GCs.

Melatonin decreases DON-induced murine ovary GC apoptosis

To determine the harmful effects of DON and the protect effect of melatonin on DON-induced cell cytotoxicity in ovary GCs, we tested the apoptotic rate of GCs pretreated with DON (2 μM), Melatonin (1.0 μM), and DON (2 μM) + Melatonin (1.0 μM) for 24 hours. DON significantly increased the ratio of apoptotic cells compared with that of the control group (control: 11.93 ± 0.71%; DON group: 16.73 ± 1.43%), while melatonin had no effect on the apoptotic rate (11.38 ± 0.83%) (Fig. 2a and b). Interestingly, treatment with DON + melatonin reduced the rate of cell apoptosis cells (10.38 ± 1.90%) to similar level of the control group (Fig. 2a and b). We further tested the level of several related apoptosis factors (ie. Bcl-2, Bax, caspase 3, cleaved caspase 3 and caspase 9), and found that DON significantly induced ovarian GC apoptosis as indicated by a decrease in the Bcl-2/Bax ratio and an increase in the cleaved caspase 3/caspase 3 ratio (Fig. 2c and d). Co-treatment with melatonin ameliorated these effects compared with those in the DON group, i.e., it restores the levels of Bcl-2, Bax, cleaved caspase 3 as well as Bcl-2/Bax and cleaved caspase 3/caspase 3 ratio to a degree similar to control (Fig. 2c and d). Moreover, we also measured the protein level of functional genes linked to proliferation, such as PCNA, CDK1, and CCND2. The result showed that DON exposure exhibited reduced levels of CDK1 and CCND2, and co-treatment with melatonin ameliorated these effects compared with those in the DON group (Fig. 2e and f). Although the alterations of PCNA levels were not significant, its trend was consistent with that of CDK1 and CCND2. Taken together, our results demonstrated that melatonin treatment ameliorated DON-induced apoptosis and facilitated the proliferation of murine ovary GCs.

Melatonin decreases ROS production in DON-exposed mouse ovary GCs

DON can induce oxidative stress in many cell types to impair cellular function [11]. Therefore, we examined the ROS levels in the GCs after DON exposure for 24 hours. The result showed that DON induced oxidative stress in ovary GCs. The ROS signal was significantly increased in the DON exposure group compared with that in the control group, while the ROS levels in the Melatonin and DON+ Melatonin groups were similar to those in the control group (Fig. 3a). Moreover, ROS relative fluorescence intensity analysis indicated that the addition of melatonin decreased the ROS production level significantly compared with that in the DON group (control: 1.00 ± 0.07; Melatonin: 0.71 ± 0.05; DON: 1.77 ± 0.11; Melatonin +DON: 0.94 ± 0.06) (Fig. 3b). To further determine the antioxidative effect of MEL in ovary GCs upon DON exposure, we tested the mRNA expression of *Sod* and *Gshpx* which are encode the main antioxidant enzymes superoxide dismutase and glutathione peroxidase. Compared with the control group, GCs treated with DON exhibited elevated levels of *Sod* mRNA expression (8.91± 0.62 *vs*. 1.01 ± 0.11, $p < 0.01$), while its expression in the melatonin + DON group was downregulated significantly (1.03 ± 0.13, $p < 0.01$), which was not different from its expression in the melatonin (Melatonin: 0.90 ± 0.04) and control groups (Fig. 3c). The expression trend of *Gshpx* was similar to that of *So*d (DON: Control: Melatonin, 2.63 ± 0.16 *vs*. 1.01 ± 0.07 *vs*. 0.85 ± 0.05, $p < 0.01$; Melatonin +DON: DON, 1.45 ± 0.12 *vs*.

2.63 ± 0.16, p <0.05) (Fig. 3d). These results illustrated that melatonin alleviated oxidative stress in murine ovary GCs induced by DON.

Melatonin ameliorates DON-induced mitochondrial dysfunction

To further verify the protective effect of melatonin on mitochondria in murine ovary GCs upon DON exposure, the $\Delta\Psi m$ and ATP content in GCs were measured after different treatments. The result showed that the addition of DON increased the level green fluorescence (Fig. 3d, DON) compared with the control group (Fig. 3d, Control) and Melatonin group (Fig. 3d, MEL), while the addition of melatonin decreased the level green fluorescence (Fig. 3d, DON + Melatonin) compared with the DON group. Meanwhile, we quantified the alteration in $\Delta\Psi m$ using the ratio of red fluorescence intensity to green fluorescence intensity (Fig. 3e). After DON treatment, the ratio decreased significantly compared with that of the control group (2.83 ± 0.17 *vs*. 3.92 ± 0.19, $p < 0.01$), while co-treatment with melatonin and DON mitigated the effect significantly (3.91 ± 0.18 *vs*. 2.83±0.17, $p < 0.01$). Moreover, DON decreased the ATP content significantly in ovary GCs compared with that in the control group and Melatonin group (2.14 ± 0.13 *vs*. 3.05±0.14 *vs*. 3.48 ± 0.28), while these effects were mitigated by co-treatment with melatonin and DON (3.23 ± 0.21 *vs*. 2.14 ± 0.13, $p < 0.05$) (Fig. 3f). These results showed that melatonin alleviated the mitochondrial dysfunction in ovarian GCs induced by DON.

Melatonin inhibits DON-induced inflammatory cytokine gene expression

DON treatment can cause an inflammatory response in many cell types. [28] To evaluate the protective effect of melatonin on the inflammatory response induced by DON treatment, the mRNA expression levels of *Tnfa* (tumor necrosis factor alpha), *Il6* (interleukin 6), and *Il1β* (interleukin 1 beta) were measured using qRT-PCR. *Tnfa*, *Il6*, and *Il1b* levels in DON-induced ovarian GCs were upregulated significantly compared with those in the control group and Melatonin group, while this effect was mitigated significantly by the addition of melatonin (Fig. 4a). Moreover, Toll-like receptor 4 (TLR4) signaling plays a vital role in the inflammatory response [29]; therefore, we investigated whether DON and melatonin affect TLR4 signaling using western blotting. The results indicated that melatonin inhibited the activation of TLR4 induced by DON in murine ovary GCs ($p < 0.05$, Fig. 4b and c).

Melatonin inhibits DON-induced downregulation of reproduction hormone genes

Considering the important physiological functions of ovary GCs, we measured the expression of reproduction hormone genes in murine ovary GCs after different treatments. The results showed that DON significantly downregulated the expression levels of *Ar* (androgen receptor), *Fshr* (follicle-stimulating hormone receptor), *Star* (steroidogenic acute regulatory protein), *P450scc* (cytochrome P450 family 11 subfamily A member 1), and *P450arom* (cytochrome P450 family 19 subfamily A member 1), while co-treatment with melatonin and DON significantly inhibited those alterations induced by DON (Fig. 5a). Meanwhile, the protein levels of AR and FSHR were measured using western blotting, the results of which were consistent with the mRNA expression of levels *Ar* and *Fshr* (Fig. 5b and c). These results indicated

that melatonin inhibits DON-induced downregulation of reproduction hormone gene expression in ovary GCs.

Melatonin reduces DON-induced inflammatory response through NF-κB and MAPK signaling pathways

To further study the anti-inflammatory effect and mechanism of melatonin on the DON-induced inflammatory response in ovary GCs, nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathway proteins were examined using western blotting. In the NF-κB signaling pathway, the levels of phosphorylated-p65 and phosphorylated-IκB were increased significantly in the DON-induced ovarian GCs compared with those in the control group and melatonin group. Interestingly, these changes were dramatically inhibited by the addition of melatonin (Fig. 6a and b). Moreover, DON administration caused a significantly increase in the phosphorylation of extracellular regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPKs, indicated the MAPK phosphorylation can be induced by DON. Meanwhile, melatonin treatment blocked the phosphorylation of these MAPKs (Fig. 6c and d). These results suggested that melatonin might reduce the activation of NF-κB and phosphorylation of ERK, JNK, and p38 MAPKs in DON-induced ovarian GCs.

Discussion

In humans, the phenomena of nausea, diarrhea, and vomiting caused by moldy food are widespread. However, people are mostly unaware that DON contamination is one of causes of these hazards. DON is a type B trichothecene and is one of the most common mycotoxins found in cereals; therefore, humans are easily expose to DON through food and water, and the level of DON could be measured by urinalysis to assess the human exposure [3]. In the present study, we determined the toxic effect and possible mechanisms of DON on murine ovary GCs and investigated the administration of melatonin to alleviate cell detriments induced by DON. Our results indicated that cell viability decreased significantly and cell apoptosis increased significantly in murine ovary GCs exposed to DON, together with increased ROS, reduced of MMP and ATP, and altered mRNA expression of inflammatory cytokine genes and reproduction hormone-genes. However, melatonin administration protected ovarian GCs from these detriments induced by DON contamination by attenuating oxidative stress-induced apoptosis and the inflammatory response. Specifically, the inhibition of NF-κB activation and the phosphorylation of MAPKs might be involved in the antiinflammatory property of melatonin (Fig. 7).

In the present study, we first determined the appropriate concentration of DON that significantly inhibited the cell viability and increased the apoptosis of murine ovary GCs in an *in vitr*o experiment. The results were similar to those in DON-exposed IPEC-J2 cells [30] and DON-exposed porcine oocytes [11], and similar to the result of IPEC-J2 induced by Zearalenone [31], which is the same type of trichothecenes as DON. To further investigate the toxicity and mechanism of DON, we tested the ROS levels of ovarian GCs exposed to DON. The result showed that DON increased of the ROS levels ovarian GCs. Previous studies reported that DON could exert cytotoxicity via oxidative stress [32], and oxidative stress could induce cell apoptosis [33]. We then hypothesized that the oxidative stress induced by DON mediated the apoptosis of

ovarian GCs. Melatonin is widely recognized as a natural antioxidant and free radical scavenger [11], and several articles proved the antioxidant property of melatonin [11, 26]. Therefore, we tested the cell viability and apoptosis of ovarian GCs after co-treatment with melatonin and DON, which showed that the administration of melatonin alleviated significantly the adverse effects on cell viability and apoptosis induced by DON in murine ovary GCs. Meanwhile, the intracellular ROS level of GCs was measured using flow cytometry after cotreatment with melatonin and DON. Interestingly, our results showed that melatonin decreased the ROS production induced by DON significantly. These results confirmed our hypothesis, and indicated the potential rescue effects of melatonin on DON exposed murine ovary GCs.

To further verify the protective effect and explore potential mechanism of melatonin, mitochondrial function was analyzed in all groups. The results showed that melatonin alleviated the mitochondrial dysfunction of ovarian GCs induced by DON significantly, by mitigating the DONinduced decline of the MMP and ATP. Mitochondria are important organelles, and several studies have reported that mitochondrial function is associated with ATP generation [34], calcium homeostasis [35], ROS production [36], cytoplasmic oxidation-reduction regulation, signal transduction, and cell apoptosis. In addition, ROS are important products derived from the process of oxidative phosphorylation [37], which produces ATP as energy to cells. However, excessive ROS production triggers oxidative stress and impairs mitochondrial function [38]. DON disrupted the balance of intracellular ROS and the antioxidative capacity, leading to mitochondrial dysfunction. Moreover, the MMP is one of the important indicators that reflect mitochondrial function [22], and the decline of the MMP is a landmark event of early apoptosis. In the present study, co-treatment with DON and melatonin significantly decreased the level of intracellular ROS, and increased the MMP and intracellular ATP, suggesting that melatonin improved mitochondrial function. A previous study reported that DON inhibited mitochondrial translation, and then disrupted the MMP [39]. Furthermore, melatonin significantly decreased the mRNA expression levels of *Sod* and *Gshpx* compared with those in the control group. SOD and GSH-PX are the main antioxidant enzymes, and which are often used as the key index of the level of the antioxidant capacity [40]. Our results were similar to previous works, i.e., melatonin significantly inhibited the upregulation of *Sod* and *Gshpx* mRNA expression induced by DON in porcine oocytes [11]. The result indicated GCs maintain a higher level of oxidative stress after DON exposure, while the administration of melatonin effectively reduced the level of oxidative stress. Collectively, our results indicated that the effects of melatonin on oxidative stress might be the mechanism by it inhibits DON cytotoxicity in ovarian GCs.

Beside oxidative stress, we also tested the inflammation response induced by DON. TNF-α, IL-1β, and IL-6 are the principal proinflammatory cytokines, and their expression levels have determined in many different types of inflammatory processes, including mastitis induced by lipopolysaccharide (LPS) [29, 41], the inflammatory processes of rat testis induced by cadmium [40], and mouse sertoli cells induced by LPS [42]. In the present study, we found that the administration of melatonin abrogated the upregulation of *Tnfa*, *Il1β*, and *Il6* induced by DON in murine ovary GCs. To further verify the potential inhibitory effect of melatonin on cytokine production, we assessed the changes in NF-κB and MAPK activation. Previous studies have reported that the expression levels of pro-inflammatory mediators are modulated by the NF-κB and MAPKs signaling pathways [43]. NFκB plays a key role in cells in response to stimuli such as

stress, free radicals, and bacterial and viral antigens. NF-κB exists in the form of homo-dimeric or hetero-dimeric complexes of p50 and p65 subunits bound to IκB [44]. In response to stimuli, IκB is phosphorylated by the activated IκB kinase, making the IκBα dissociate from the trimer, and then the phosphorylated IκBα is ubiquitinated and degraded. However, the dissociated NF-κB p65 rapidly enters the nucleus from the cytoplasm, binds to specific DNA sequences in the nucleus, which triggers the expression and regulation of numerous genes that are involved in the process of immune regulation, the inflammatory response, and anti-apoptosis [45]. Herein, our results showed that the administration of melatonin blocked the phosphorylation of p65 and the degradation of IκBα induced by DON significantly. These results indicated that melatonin exerts its anti-inflammatory activity by inhibiting NF-κB by blocking the phosphorylation of p65 and the degradation of IκBα. The MAPK signaling pathway plays an important role in the regulation of cell growth, differentiation, stress, inflammation, and other important physiological and pathological effects. Herein, we analyzed the protein expression of ERK1/2, p38, and JNK, which are the three main MAPKs subfamilies. The results showed that supplementation with melatonin inhibited the upregulation of the phosphorylation status of p38, ERK1/2, and JNK induced by DON. In terms of the relationship between melatonin and inflammation, melatonin plays an important role in the immune system [17]. Carrillo-Vico et al. reviewed the multiple effects of melatonin on the immune system, pointing out the pleiotropic effects of melatonin on the regulation of the immune system [19]. Meanwhile, melatonin could inhibit the proliferation, invasion, metastasis, and angiogenesis of gastrointestinal cancer cell, promote its apoptosis and cancer immunity, and exerted and antigastrointestinal cancer effect [18]. Therefore, increasing research has focused on the application and production of melatonin because of its multiple effects. Collectively, these results demonstrated that melatonin suppressed the inflammatory response induced by DON mainly through inhibiting NFκB and MAPKs activation. Taking our results together with those of previous studies, we determined that melatonin could inhibit the pro-inflammatory cytokine production induced by DON by preventing the activation of NF-kB and MAPKs.

Conclusion

In summary, our results indicated that DON exposure caused oxidative stress, inflammationmediated apoptosis, and altered mRNA expression of reproduction hormone genes. Interestingly, administration of melatonin significantly protects murine ovary GCs from the toxicity of DON via its antioxidative and anti-inflammatory capacities. Meanwhile, some unknown pathways maybe involve in melatonin ameliorates the toxicity induced by deoxynivalenol in murine ovary Granulosa Cells. Combined with the protect effect of melatonin on oocytes upon DON exposure [11], melatonin can be regarded as a potential agent for prophylaxis and treatment of diseases induced by DON contamination.

Abbreviations

DON: deoxynivalenol; MEL, melatonin; *Ar*: androgen receptor; *Fshr*: follicle-stimulating hormone receptor; *Star*: steroidogenic acute regulatory protein; *P450scc*: cytochrome P450 family 11 subfamily A member 1;

P450arom: cytochrome P450 family 19 subfamily A member 1; *Hsf2*, heat shock factor 2; MMP: Mitochondrial Membrane Potential; ERK, extracellular regulated kinase; JNK: c-Jun N-terminal kinase; PCNA proliferating cell nuclear antigen; CDK1: cyclin-dependent kinases 1; CCND2: cyclin D2; NF-κB: nuclear factor kappa B; MAPK: mitogen-activated protein kinase.

Declarations

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Author Contributions

Wenbin Bao, Shenglong Wu and Haifei Wang participated in the experimental design. Hairui Fan and Shiqin Wang performed the experiments and data analyses. Hairui Fan and Mingan Sun wrote and revised the manuscript.

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Availability of data and materials

The data and materials supporting the conclusions are included within the article and its supplementary information files.

Ethics approval and consent to participate

All mice animal experiments were approved by the Animal Care and Use Committee of Yangzhou University.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing financial interest.

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Figures

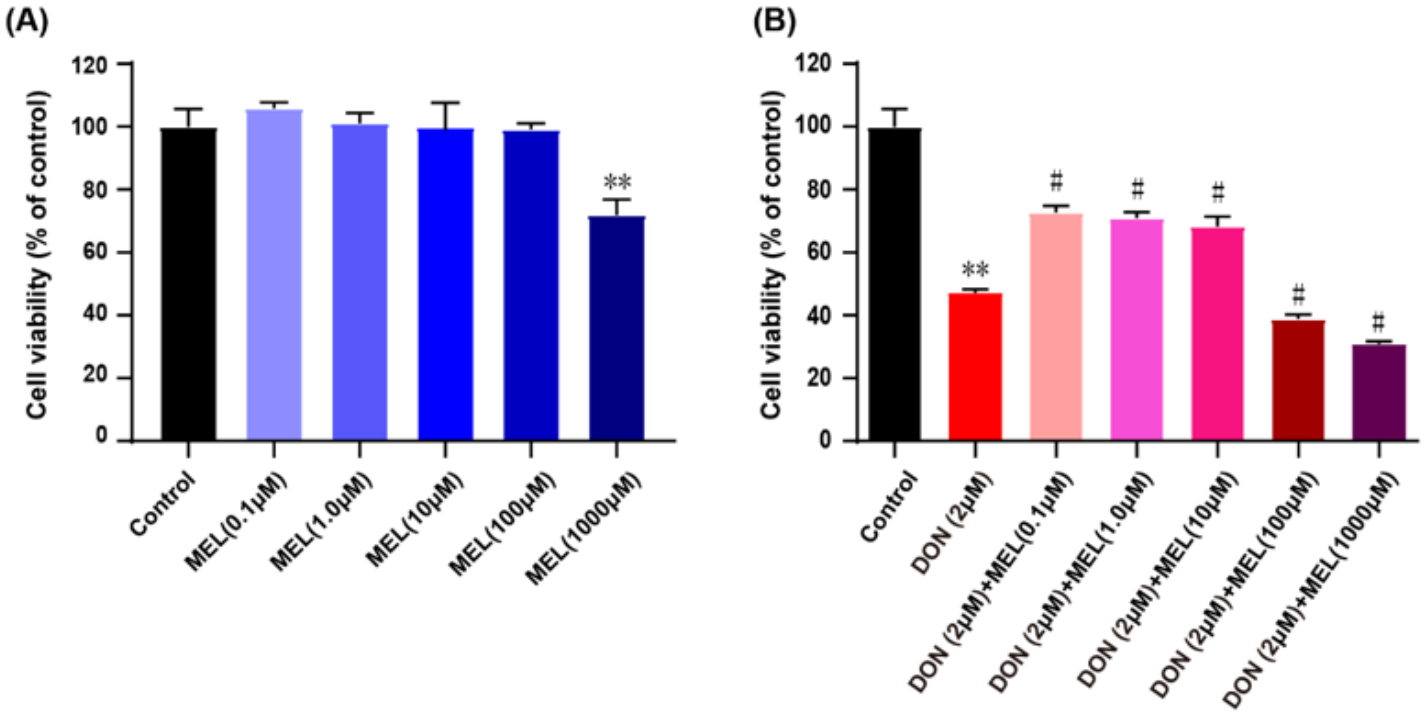

Figure 1

The effect of Melatonin (MEL) on DON-Ovary GC viability. Ovarian GCs were stimulated with DON, MEL, and DON+MEL for 24 h. Cell viability was determined using the CCK-8 assay. The data are expressed as the means ± SEM. **p < 0.05 compared with the control; #p < 0.05 compared with the DON group. The results showed that MEL significantly inhibited DON-induced decreases in cell viability.

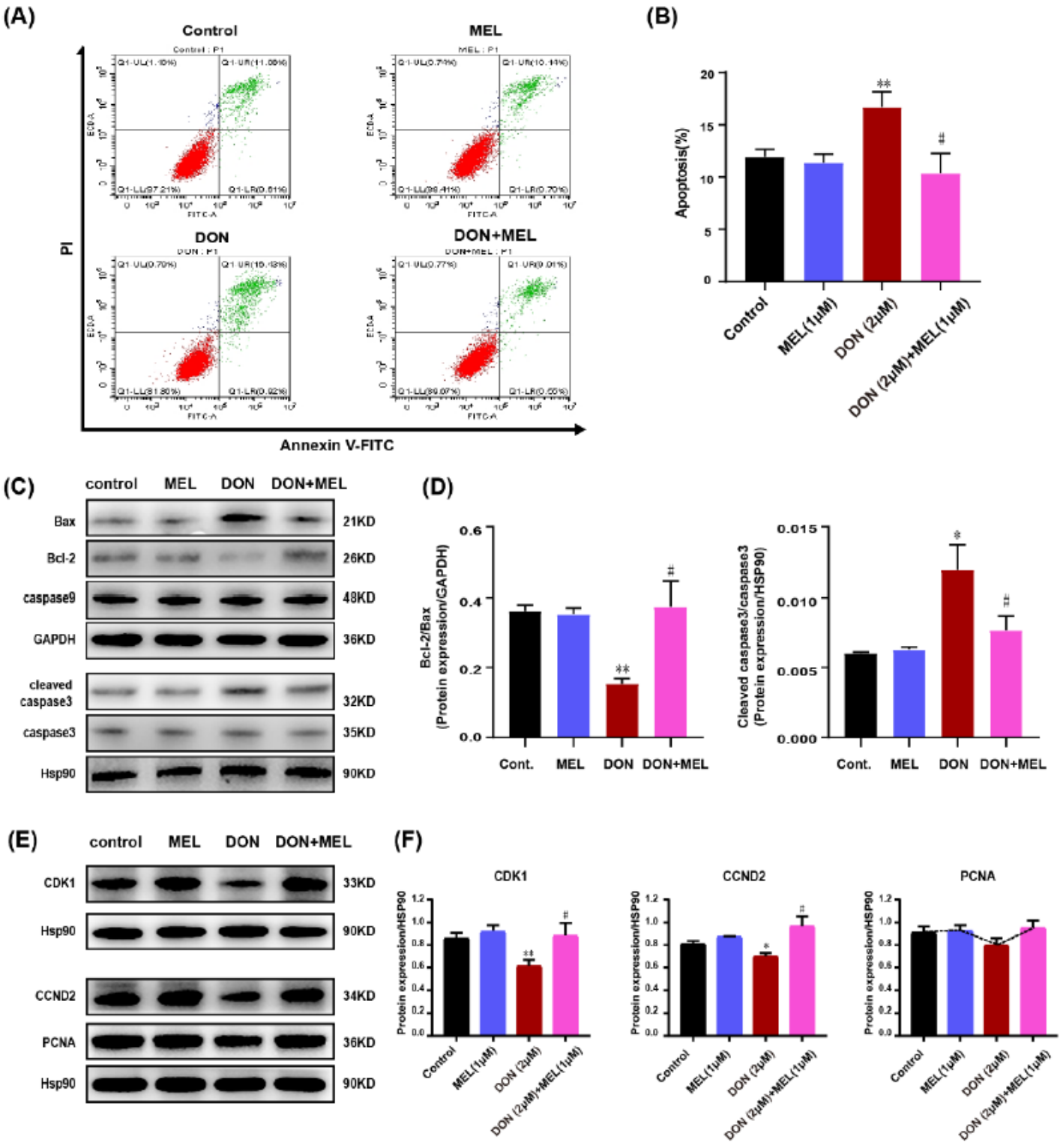

Figure 2

Melatonin treatment ameliorates DON-induced mouse ovary GC apoptosis. (a) Apoptosis of GCs was evaluated by measurement of Annexin V using flow cytometry. Apoptotic cells were Annexin V-positive and PI-negative. (b) The Figure shows representative staining, and the numbers in the quadrants indicate the percentage of cells within in the respective subpopulations. (c, d) Western blotting analysis of the changes in the protein levels of related apoptosis factors (Bax, Bcl-2, cleaved caspase-3, caspase-3,

caspase9). (e, f) Western blotting analysis of the changes in the protein level of functional genes linked to proliferation (PCNA, CDK1 and CCND2). Each bar represents the mean ±SD from three different experiments, n = 3. *p < 0.05 and **p < 0.01 compared with the control and Melatonin (MEL) groups; #p < 0.05 compared with the DON group.

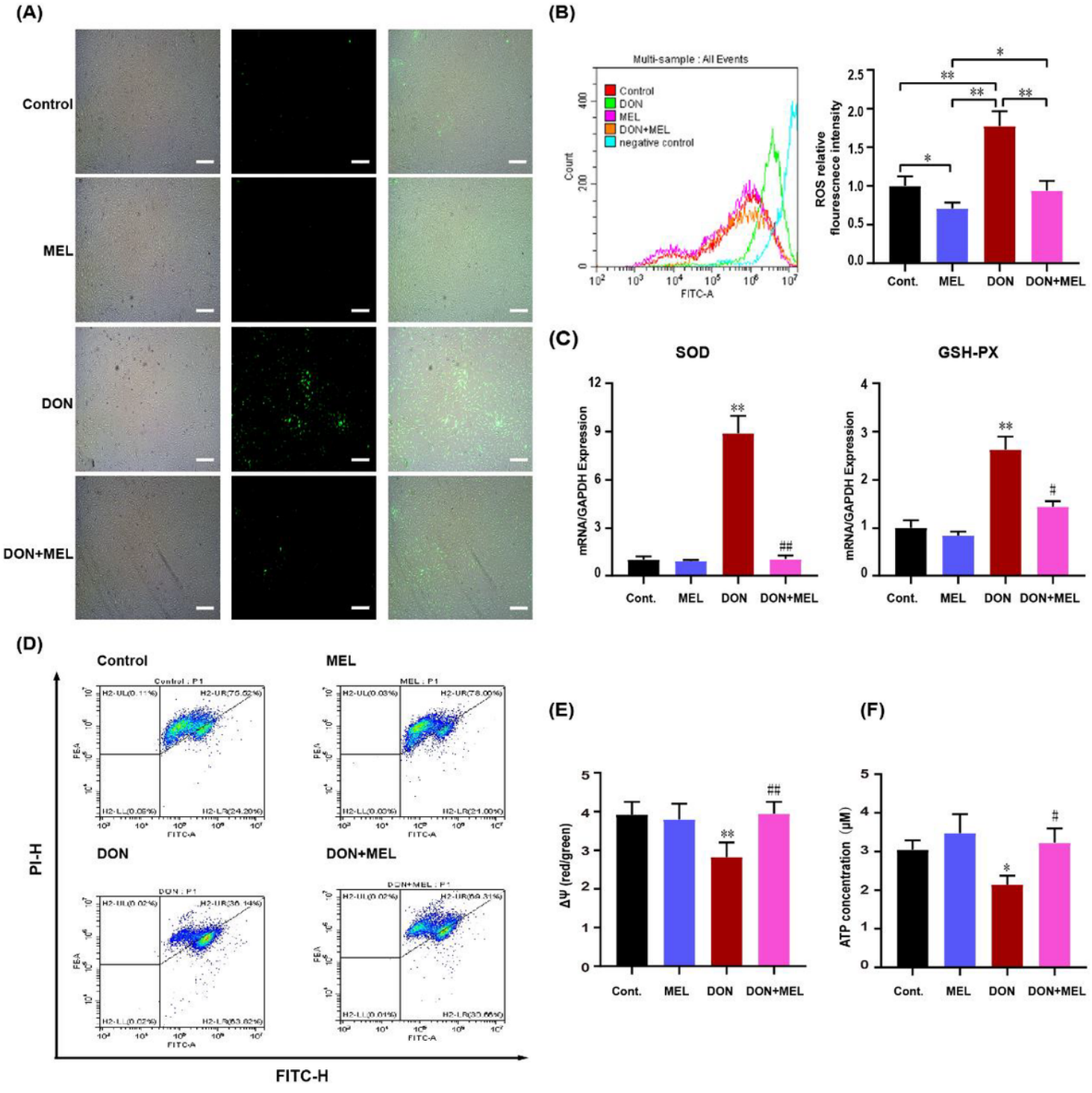

Figure 3

Effects of melatonin on ROS levels and mitochondrial function of murine ovary GCs upon DON exposure. (a) Representative images of ROS levels in the control, Melatonin (MEL)-exposed, DON-exposed, and MEL + DON-treated mouse ovary GCs. Scale bar, 300 μm; (b) Fluorescence intensities of ROS in the mouse

ovary GCs of the different groups; (c), qRT-PCR analysis of the relative expression of Sod and Gshpx mRNA levels. (d) Mitochondrial Membrane Potential levels of the different groups. (e) The ratio of the red over green fluorescence intensity by flow cytometry represents the quantitative ΔΨm in each group. (f) ATP content of the different groups. All values are expressed as the means ± SD (n = 3), **p < 0.01 versus the Control group; #p < 0.05 versus the DON group.

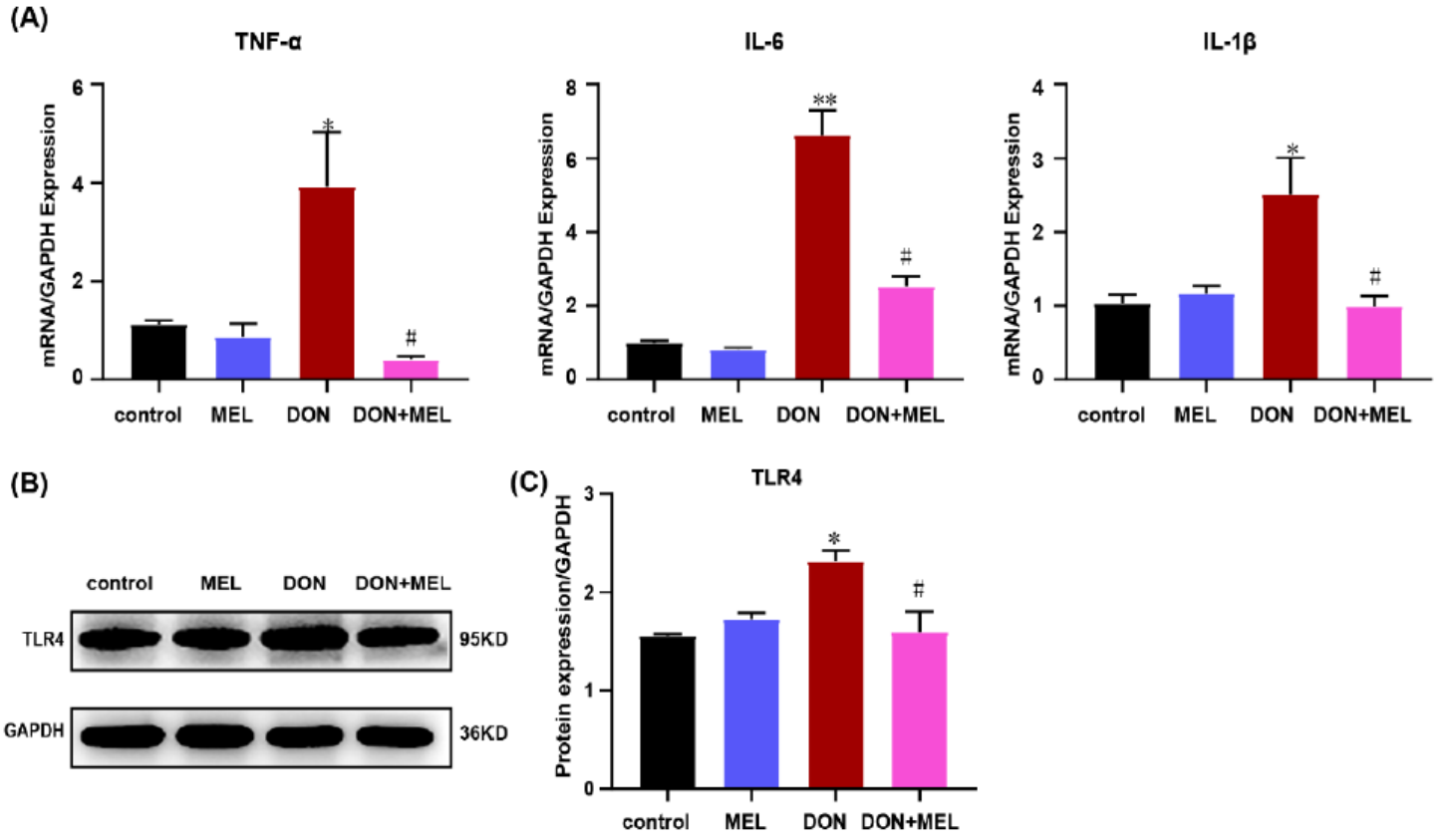

Figure 4

Expression of mRNAs encoding inflammatory cytokines in DON-exposed mouse ovary GCs measured using qRT-PCR. Ovary GCs were stimulated with DON, Melatonin (MEL), and DON+MEL treated for 24 h. (a) The relative expression kevels of Tnfa, Il6 and Il1b were calculated by dividing their values by the Gapdh mRNA expression levels. All data are expressed as the means ± SEM. (b and c) TLR4 (1:1000 dilution) protein levels were examined in GCs after different treatments. GAPDH (1:5000 dilution) was used ad internal control. *p < 0.05 and **p < 0.01 compared with the control and MEL groups; # p < 0.05 compared with the DON group.

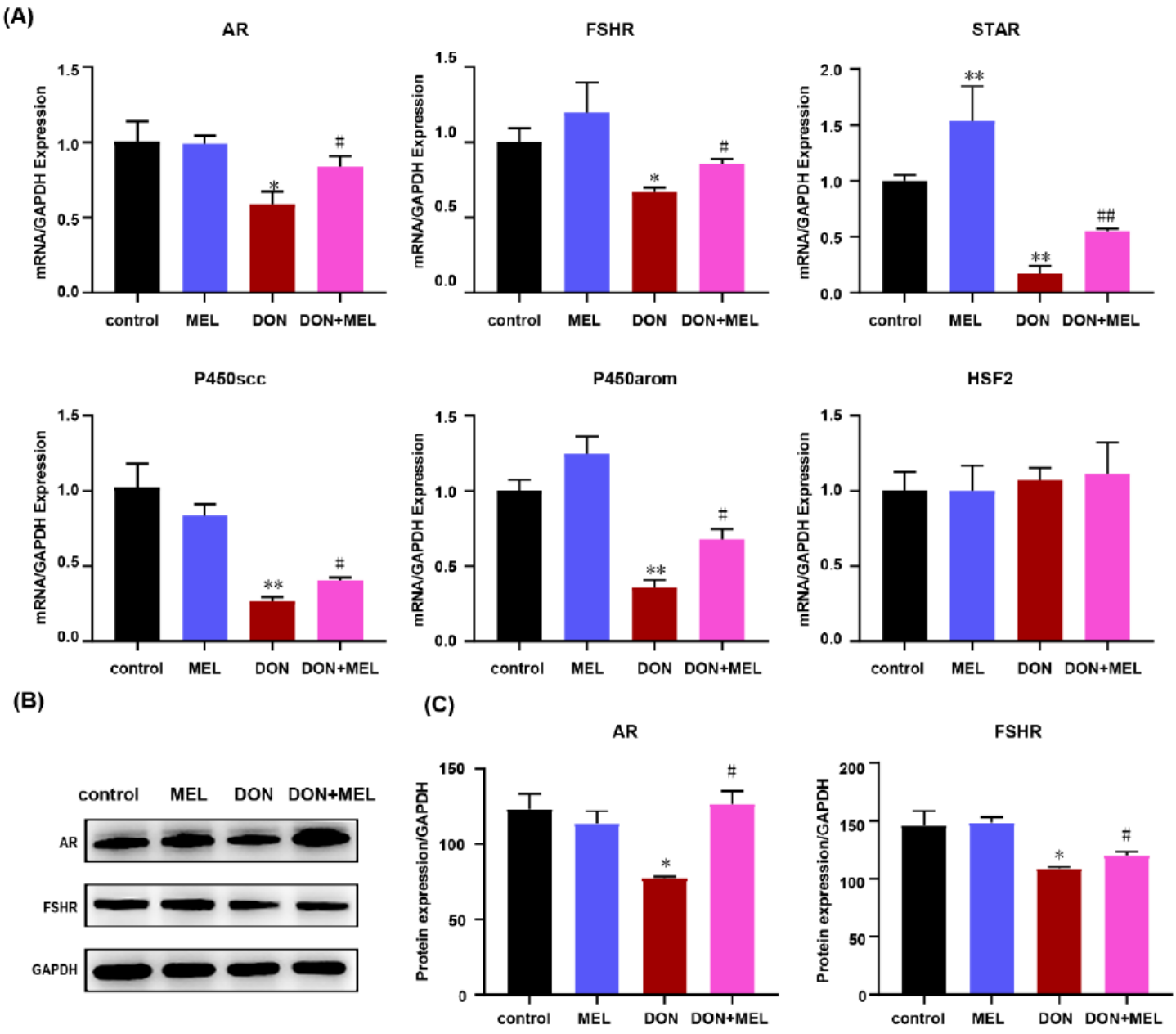

Figure 5

Effect of Melatonin on DON-induced expression of reproductive hormone-related genes in mouse ovary GCs, measured by qRT-PCR and western blotting. Ovarian GCs were stimulated with DON, Melatonin (MEL), and DON+MEL for 24 h. (a) The relative expression levels of Ar, Fshr, Star, P450scc, P450arom, and Hsf2 were calculated by dividing their values by the Gapdh mRNA expression level. All data are expressed as the means ± SEM. (b and c) The protein levels of AR (1:1000 dilution) and FSHR (1:1000 dilution) in murine ovary GCs, assessed using western blot. GAPDH (1:5000 dilution) as the control. The results from each group are shown as the mean ± SD, n = 3. *p < 0.05 and **p < 0.05 compared with the Control and MEL groups; # p < 0.05 and ## p < 0.05 compared with the DON group.

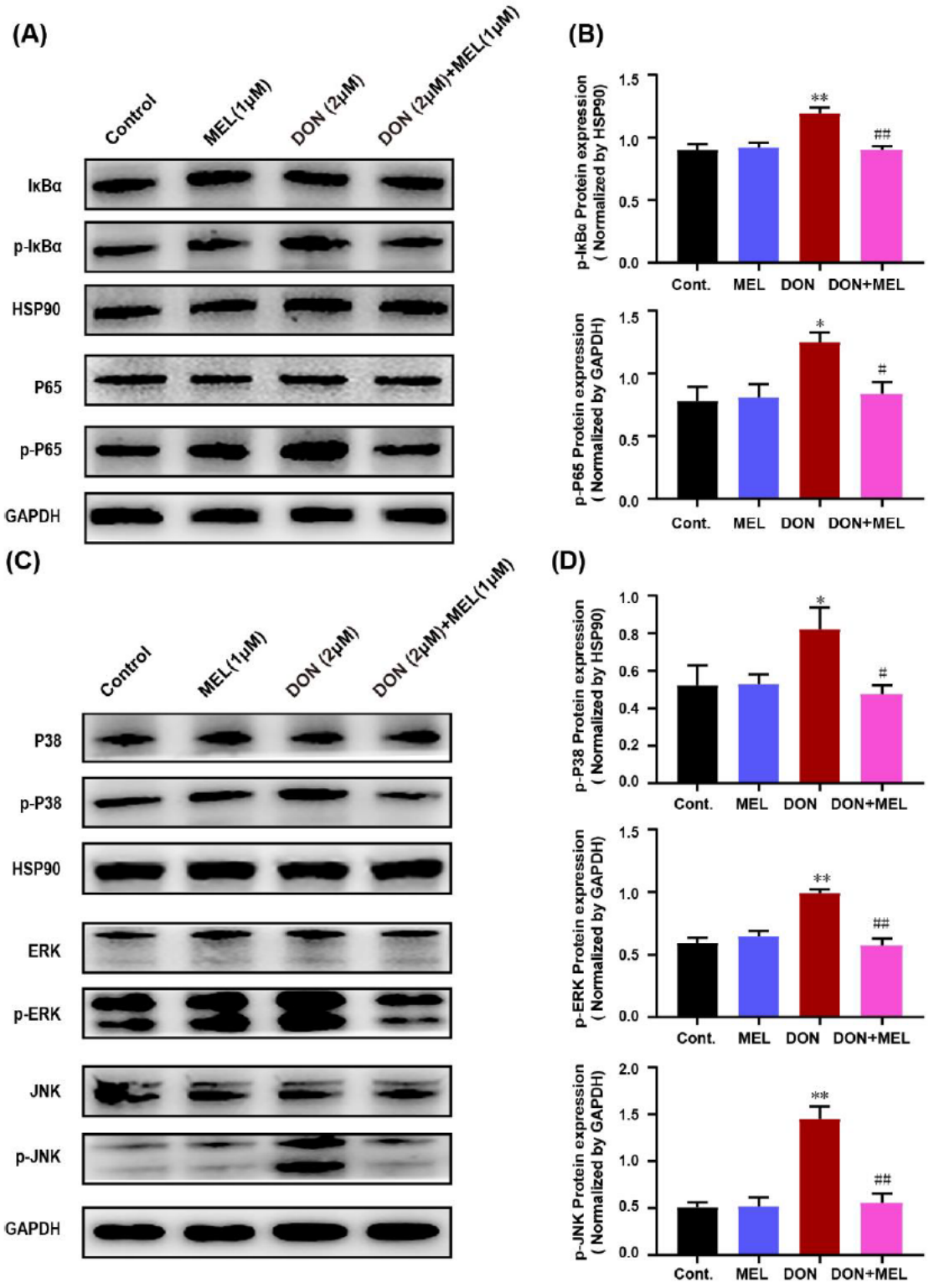

Figure 6

Melatonin (MEL) inhibits DON-induced NF-κB and MAPK activation in murine ovary granulosa cells. Ovarian GCs were stimulated with DON, MEL, and DON+MEL for 24 h. (a and b) IkBα (1:1000 dilution), p-IκBα (1:1000 dilution), P65 (1:1000 dilution), and p-P65 (1:1000 dilution) were examined using western blotting in GCs treated with DON, MEL, and DON+MEL for 24 h. (c and d) P38 (1:1000 dilution), p-P38 (1:1000 dilution), ERK (1:1000 dilution), p-ERK (1:1000 dilution), JNK (1:1000 dilution), and p-JNK (1:1000

dilution) were examined by western blotting in GCs. All data are shown as mean ± SD, n = 3. *p < 0.05 and ** p< 0.05 compared with the Control and MEL groups; # p < 0.05 and ## p < 0.05 compared with the DON group.

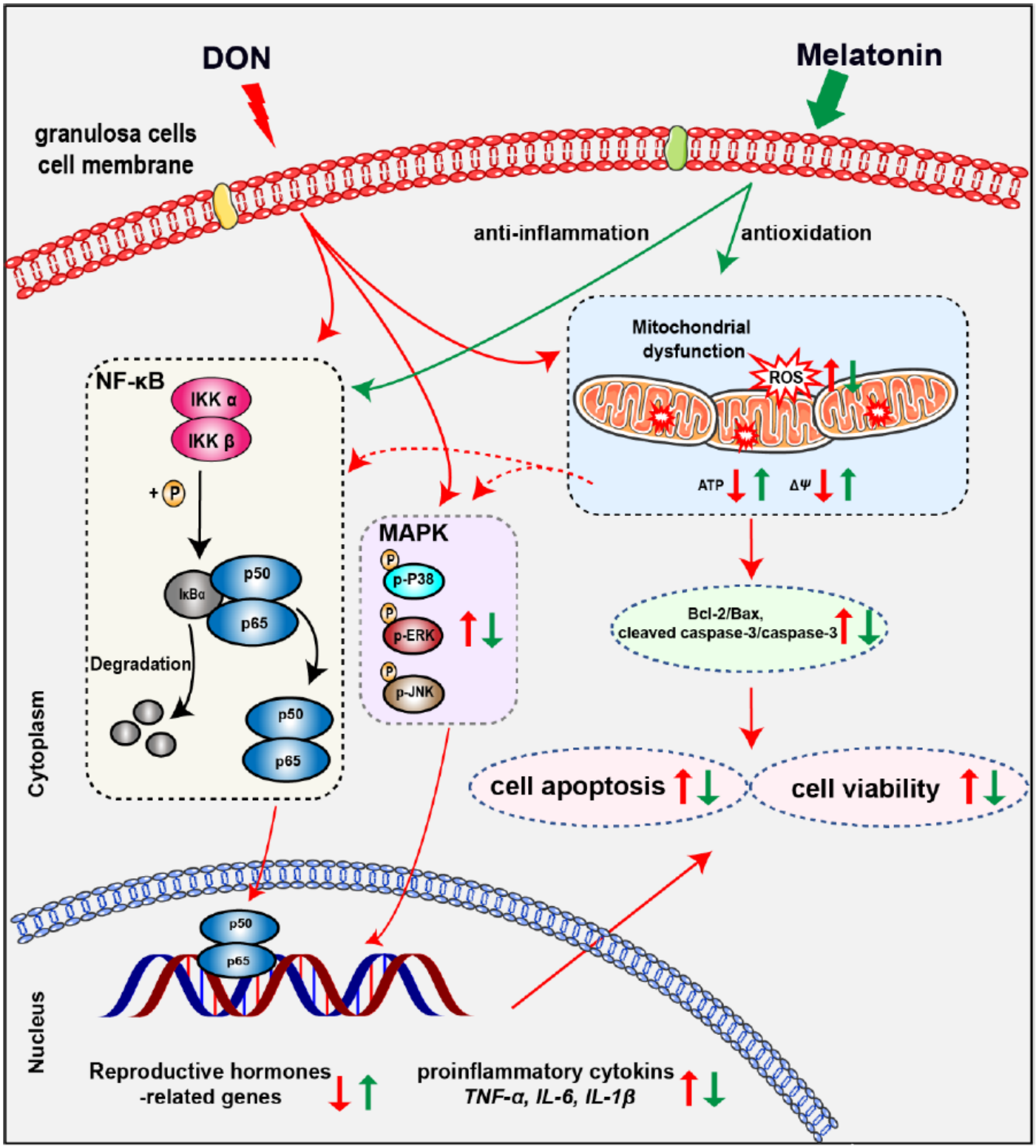

Figure 7

Diagram of DON exposure and melatonin administration to murine ovary granulosa cells.

Supplementary Files

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