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Melatonin protects against lipopolysaccharide-induced epididymitis in sheep epididymal epithelial cells *in vitro*

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ABSTRACT

Melatonin has protective effects against inflammation but its role in epididymitis is unknown. We addressed this in the present study using lipopolysaccharide (LPS)-stimulated sheep epididymal epithelial cells as an *in vitro* inflammation model. We found that interleukin (IL)-1β, IL-6, tumor necrosis factor α, and cyclooxygenase (COX)-2 mRNA levels; COX-2 and Toll-like receptor (TLR)-4 protein levels; and nuclear factor (NF)-κB p65 phosphorylation were increased by LPS treatment. These effects were reversed in a dose-dependent manner by melatonin (10^{-11}–10^{-7} M). Quantitative reverse transcription PCR and immunofluorescence analyses showed that the melatonin receptors MT1 and MT2 were expressed in sheep epididymal epithelial cells. The inhibitory effect of melatonin on inflammation was abrogated by the MT1 and MT2 receptor antagonist luzindole and the MT2 ligand 4-phenyl-2-propanamide tetraldehyde. Thus, melatonin exerted anti-inflammatory effect in epididymal epithelial cells by inhibiting TLR4/NF-κB signaling, suggesting its potential as an effective drug for the treatment of epididymitis in sheep.

1. Introduction

The epididymis is the site of sperm development, maturation, transportation, and storage. Epididymitis is one of the most common diseases in most mammals and human males [1,2]. Although the exact etiology and pathogenesis are unknown, inflammatory cytokines including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α are thought to play a key role [3–6]. For instance, the mRNA levels of IL-1β, IL-6, IL-10, and TNF-α were found to be upregulated in lipopolysaccharide (LPS)-induced epididymitis in rats [7]. Current treatments for epididymitis include immunological drugs and broad-spectrum antibiotics, but their side effects diminish the quality of life of patients. As such, there is a need for alternative therapies.

Melatonin (n-acetyl-5-methoxytryptamine) was initially identified in the pineal gland as an amine hormone [8] that is secreted into the circulation, and is known to have diverse biological functions including antioxidant, anti-inflammatory, anti-tumor, and immunoregulatory activities [9–13]. Melatonin inhibits inflammation via two mechanisms. One is receptor-independent and is attributable to its capacity to eliminate free radicals [14]: melatonin and its metabolites effectively remove hydroxyl free radicals and peroxynitrite [15–21], and melatonin was shown to prevent tissue injury in mouse testicles by reducing oxidative stress and inflammation [22–24], suggesting that it indirectly regulates the inflammatory response by acting as an antioxidant.

A second anti-inflammatory mechanism of melatonin involves membrane melatonin receptor (MT)1 and MT2, which suppress the overproduction of inflammatory cytokines [25,26]. Melatonin enhanced the resistance of pancreatic tissue to inflammatory mediators by activating MT2 [27] and in LPS-stimulated human vascular smooth muscle cells, melatonin inhibited inflammatory cytokine production [28]. It is thought that this mechanism prevents the activation of nuclear factor (NF)-κB by Toll-like receptor (TLR)4 [28,29]. In fact, it was reported that melatonin decreased 2,4,6-trinitrobenzene sulfonic acid-induced colonic inflammation in rats by blocking NF-κB signaling [30]. Many studies have explored the therapeutic potential of melatonin, given that its hydrophilicity and lipophilicity allow its passage through the blood–testis barrier into the testis [31]. In addition, melatonin is known to inhibit the expression of TLR4, activation of NF-κB, and

Abbreviations: 4P-PDOT, 4-phenyl-2-propanamide tetraldehyde; COX-2, cyclooxygenase 2; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IL, interleukin; LPS, lipopolysaccharide; MT, melatonin receptor; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; PE, phycoerythrin; qRT-PCR, quantitative reverse transcription PCR; RPMI1640, Roswell Park Memorial Institute 1640; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor

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expression of inflammatory factors in LPS-induced CRL 1999 and RAW264.7 cells [28,29]. To the best of our knowledge, little information is available regarding the effect of melatonin on TLR4 expression and NF-κB activation in epididymal epithelial cells.

To address this issue, the present study investigated the effect of melatonin on LPS-treated sheep primary culture epididymal epithelial cells. The aim was to determine whether melatonin is an effective anti-inflammatory agent for the treatment of sheep epididymal epithelium inflammation, and to provide a reference for further studies on the anti-inflammatory effect of melatonin against sheep epididymitis.

2. Materials and methods

2.1. Reagents

LPS was purchased from Solarbio (Beijing, China). Melatonin was from TargeMol (Shanghai, China). Roswell Park Memorial Institute (RPMI)1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were from Hyclone (Logan, UT, USA). Antibodies against cyclooxygenase (COX)-2, p65, phosphorylated (p-)p65, MT1, MT2, and β-actin and horseradish peroxidase-conjugated anti-rabbit IgG were from Bioss (Beijing, China). Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG and phycoerythrin (PE)-conjugated anti-rabbit IgG were from TransGen (Beijing, China). Anti-TLR4 antibody was from Proteintech (Rosemont, IL, USA). Luzindole and 4-phenyl-2-propanamide tetraldehyde (4P-PDOT) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Melatonin solution was prepared according to the manufacturer's instructions (TargeMol Shanghai, China), in which the stock solutions (10 mM melatonin) were prepared in dimethyl sulfoxide (DMSO). The subsequent dilutions were prepared using the basal culture medium depending on the protocol used. The final concentration of the solvent was ≤ 0.1% (v/v), which had no effect on the experiments [32].

2.2. Culture of sheep epididymal epithelial cells

Experimental procedures were approved by the Animal Care and Use Committee of the College of Veterinary Medicine of Gansu Agricultural University. Sheep epididymal epithelial cells were harvested and cultured as previously described [33]. Briefly, healthy sheep testicles were collected from the Xiaoxihu slaughterhouse in Lanzhou, Gansu Province, and immediately immersed in phosphate-buffered saline (PBS) containing antibiotics and transported to the laboratory within 20 min. The epididymal tissue was separated on a clean workbench and the caudal portion was cut into ~ 1-mm^3 pieces that were washed with PBS. The tissue was incubated in RPMI1640 medium containing 0.2% collagenase type 4 (Sigma-Aldrich) at 37 °C for 1 h. Dissociated cells were separated from undigested tissue by passage through a 150-mm stainless steel strainer. Cells were resuspended in RPMI1640 medium supplemented with penicillin (100 IU/ml), streptomycin (100 IU/ml), hydrocortisone (10^{-7} M) (Sigma-Aldrich), insulin (10^{-7} M) (Sigma-Aldrich), transferrin (5 μg) (Bioss), and 10% (v/v) FBS after washing with culture medium. The medium was changed every 2 days. After 5 days of culture, cells had reached 80%–90% confluence and were seeded in 6-well plates (Corning Inc., Corning, NY, USA) containing 2 ml of culture medium; these were placed in a humidified incubator at 37 °C with 95% O_2/5% CO_2 for 24 h. Sheep epididymal epithelial cells were cultured in serum-free RPMI1640 medium for 12 h.

2.3. Immunofluorescence analysis

MT1 and MT2 protein expression in sheep epididymal epithelial cells was detected by immunocytochemistry. Briefly, the cells were fixed in 4% (v/v) paraformaldehyde, washed with ice-cold PBS, and treated with 0.1% (v/v) Triton X-100 for 30 min. After incubation with 5% (w/v) bovine serum albumin for 30 min, the cells were incubated overnight at 4 °C with a rabbit polyclonal antibody against MT1 (bs-0027R, 1:100) or MT2 (bs-0963R, 1:100). The primary antibodies were detected by incubation at 37 °C for 45 min with FITC-conjugated goat anti-mouse IgG (H + L) (1:200) and PE-conjugated goat anti-rabbit IgG (H + L) (1:200), respectively. Nuclei were counterstained with 1 μg/ml of 4′,6-diamidino-2-phenylindole (Solarbio). Digital images were acquired on a DP73 optical microscope (Olympus, Tokyo, Japan).

2.4. Cell treatments

To investigate the effect of melatonin on the expression of IL-1β, IL-6, COX-2, TNF-α, TLR4, and NF-κB p65 in sheep epididymal epithelial cells stimulated with LPS, cells were cultured with LPS at a final concentration of 10 ng/ml for 24 h (LPS treatment group); some of these cells were cultured with three different final concentrations of melatonin (10^{-11}, 10^{-9}, and 10^{-7} M) for 24 h (LPS + melatonin groups). The cells were harvested for mRNA or protein expression analysis.

To investigate the mechanism underlying the effect of melatonin on epididymitis, epididymal epithelial cells were treated with the non-selective MT1 and MT2 receptor antagonist luzindole or the selective MT2 ligand 4P-PDOT. After changing the medium, melatonin (10^{-7} M) and luzindole (10^{-7} M) or 4P-PDOT (10^{-7} M) were added, followed by incubation for 24 h. The cells were then harvested for mRNA or protein expression analysis.

2.5. Total RNA isolation and quantitative reverse transcription (qRT-)PCR

Total RNA was extracted from cells using TRIzol reagent (Solarbio) and reverse-transcribed into cDNA using the Prime Script RT Reagent Kit with gDNA Eraser (Takara Bio, Dalian, China). IL-1β, IL-6, COX-2, and TNF-α mRNA levels were evaluated by qRT-PCR on a LightCycler 480 Realtime Detection System (Roche, Basel, Switzerland). Reaction mixtures consisted of 10 μl of 2 × SYBR Green II PCR Mix (Takara Bio), 25 μmol/l forward and reverse primers, 2 μl of template, and double-distilled H_2O up to a final volume of 20 μl. The reactions conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 10 s. A melting curve was obtained from 65 °C to 95 °C, increasing in increments of 0.5 °C every 5 s. β-Actin was used as an internal reference gene. Table 1 shows the sequences of forward and reverse primers used in this experiment. Expression levels of target genes were calculated as relative values with the $2^{-\Delta\Delta CT}$ method. PCR products were visualized by 2% agarose gel electrophoresis.

Table 1
Primers used for real-time reverse transcription PCR.

Gene	Sequences (5'→3')	Product length (bp)	Accession no.
IL-1β	F: TCTCCCTAAAGAAGCCATAC R: AGAGCGTCTCAGCACGAATA	153	NM_001009465.2
IL-6	F: GCAGACTACTTCTGACCACTCCA R: TTTTCACACTCGTCATTCTTCTCAC	112	NM_001009392.1
COX-2	F: TGAGGAACTTACAGGAGAGAAGGAA R: TACCAGAAGGGCGGGATACA	94	NM_001009432.1
TNF-α	F: GGGAACACAGACAGAGGGGACA R: CCTGCGAGTAGATGAGGTAAAG	260	EF446377.1
MT1	F: AGCACGAATTCCCTCTGCTA R: GAGCATCGGAACGATGAAAT	183	NM_001009725.1
MT2	F: AGGTCAAGGCGGAGAGCAA R: GCCACTTCTTCGGGGTCAA	148	NM_001130938.1
β-actin	F: GTCACCAACTTGGGACGACA R: AGGCGTACAGGGACAGCA	208	U39357

2.6. Western blotting analysis

Epididymal epithelial cells were treated with ice-old radioimmunoprecipitation assay lysis buffer containing 1 mmol/l phenylmethylsulfonyl fluoride. Cell lysates were centrifuged at 12,000 × *g* at 4 °C for 5 min. Protein concentrations were determined with the BCA Protein Assay Kit (Solarbio). Equivalent amounts of protein (40 μg) were loaded onto a gel and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and then electrotransferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, US) that was blocked with 5% skimmed milk in Tris-buffered saline/Tween 20 at room temperature for 1 h. The blocked membrane was incubated overnight at 4 °C with rabbit polyclonal antibodies against COX-2 (bs-0732R, 1:500), TLR4 (19811-1-AP, 1:1000), p65 (bs-0465R), p-p65 (bs-0982R), and β-actin (bs-0061R, 1:3000). After washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (bs-0295G-HRP; 1:3000 dilution). Immunoreactivity was visualized by enhanced chemiluminescence (Abnova, Taipei, Taiwan) and signals were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.7. Statistical analysis

Statistical analyses were performed using SPSS v.19.0 software (IBM, Armonk, NY, USA). Quantitative data are presented as mean ± SEM. All data were tested for normality and homoscedasticity. Differences between groups were evaluated by one-way analysis of variance followed by Duncan's multiple range test. $P < 0.05$ was considered significant.

3. Results

3.1. Melatonin suppresses LPS-induced inflammation in sheep epididymal epithelial cells

To investigate the anti-inflammatory effect of melatonin on sheep epididymal epithelial cells, we evaluated IL-1β, IL-6, COX-2, and TNF-α mRNA levels by qRT-PCR following LPS treatment. IL-1β, IL-6, COX-2, and TNF-α expression was upregulated by LPS treatment relative to the control group; however, this effect was reversed in a dose-dependent manner by melatonin ($P < 0.05$, Fig. 1A, B).

3.2. Melatonin inhibits TLR4 expression and NF-κB activation in LPS-treated epididymal epithelial cells

To investigate the anti-inflammatory mechanism of melatonin, we examined TLR4 and NF-κB activation in LPS-treated cells. The levels of both TLR4 and NF-κB were increased relative to the control group by LPS; however, a dose-dependent decrease in the levels was observed in the presence of melatonin ($P < 0.05$, Fig. 2).

3.3. MT1 and MT2 are expressed in sheep epididymal epithelial cells

To further examine the action of melatonin, we analyzed the expression of MT1 and MT2 in sheep epididymal epithelial cells. MT1 and MT2 transcripts were detected in the cells by qRT-PCR (Fig. 3A), and immunofluorescence analysis revealed positive signals in the cytoplasm and at the cell membrane (Fig. 3B).

3.4. Effects of melatonin and luzindole or 4P-PDOT on inflammation-related genes expression in LPS-treated epididymal epithelial cells

To determine whether the inhibitory effect of melatonin on LPS-induced inflammation in epididymal epithelial cells involves a receptor-dependent mechanism, we incubated LPS-treated cells with melatonin and the MT1/MT2 antagonist luzindole or the MT2 antagonist 4P-PDOT. The results showed that both the non-selective antagonist luzindole (10^{-7} M) and MT2-selective antagonist 4P-PDOT (10^{-7} M) mitigated the inhibitory effect of melatonin on the expression of the inflammatory cytokines IL-1β, IL-6, COX-2, and TNF-α, with a greater effect relative to the LPS + melatonin (10^{-7} M) group observed in cells treated with melatonin and luzindole as compared to melatonin and 4P-PDOT ($P < 0.05$, Fig. 4A, B). These results suggest that both MT1 and MT2 mediate the anti-inflammatory effects of melatonin.

3.5. Effects of melatonin and luzindole or 4P-PDOT on LPS-induced TLR4 and NF-κB signaling in epididymal epithelial cells

To evaluate whether the inhibitory effect of melatonin on the TLR4 and NF-κB pathways in LPS-induced epididymal epithelial cells is dependent on the mechanisms of its receptors, MT1 and MT2, we incubated sheep epididymal epithelial cells with melatonin plus luzindole or 4P-PDOT and evaluated the expression of these signaling molecules. We found that both luzindole (10^{-7} M) and 4P-PDOT (10^{-7} M) abrogated the inhibitory effect of melatonin on TLR4 and NF-κB activation, with cells treated with melatonin + luzindole showing a more pronounced effect than those treated with melatonin + 4P-PDOT compared to the LPS + melatonin (10^{-7} M) group ($P < 0.05$, Fig. 5).

4. Discussion

Epididymitis is a common disease of the reproductive system that is detrimental to male reproductive health and causes male sterility or reduced spermatogenesis [2]. Melatonin is known to be an effective anti-inflammatory agent [34–36]. In this study, we showed that melatonin suppressed LPS-induced production of inflammatory cytokines in a concentration-dependent manner, and blocked the expression of TLR4 as well as activation of NF-κB signaling, leading to a decrease in inflammatory cytokine levels. These anti-inflammatory effects were mediated via MT1 and MT2. Our results are consistent with the findings of studies on melatonin in rats [6,25–30].

The innate immune system recognizes pathogens through pattern recognition receptors such as TLR4, which plays a key role in inflammation. LPS is recognized by TLR4 on the cell membrane and this leads to the activation of NF-κB signaling. NF-κB is a nuclear transcription factor that regulates the expression of IL-1β, TNF-α, and other inflammatory cytokines [37]. We therefore examined TLR4 expression and NF-κB activation in LPS-treated cells in order to elucidate the mechanism by which melatonin inhibits LPS-induced inflammation and found that both were reduced. These results are in agreement with previous studies on macrophages [29] and indicate that TLR4 is involved in the regulation of epididymitis in sheep. It was previously shown that NF-κB activity is higher in rats with colitis than in healthy controls; moreover, melatonin suppresses NF-κB activity, thereby reducing inflammation-related injury [30]. This is consistent with the results of this study, indicating that LPS stimulation of sheep epididymal epithelial cells can cause the activation of the NF-κB pathway and may be involved in this inflammatory process, and that melatonin had a significant inhibitory effect on this inflammation. Thus, we conclude that melatonin reduced inflammation by inhibiting TLR4 expression and NF-κB activation, thereby suppressing pro-inflammatory cytokine production.

The physiological functions of melatonin involve multiple pathways and partly depend on its receptors, MT1 and MT2 [38]. In this study, we detected MT1 and MT2 expression in caudal epididymal cells by immunofluorescence microscopy and RT-PCR. The results showed that the epididymis may be a target organ of melatonin, providing an indirect evidence of the regulatory effect of melatonin on epididymis function. To investigate the role of melatonin receptor-dependent mechanisms in epididymal function, we used the non-selective MT1 and MT2 receptor antagonist luzindole [39] and the selective MT2 ligand 4P-PDOT [40,41]. In this study, we found that blocking these receptors abolished

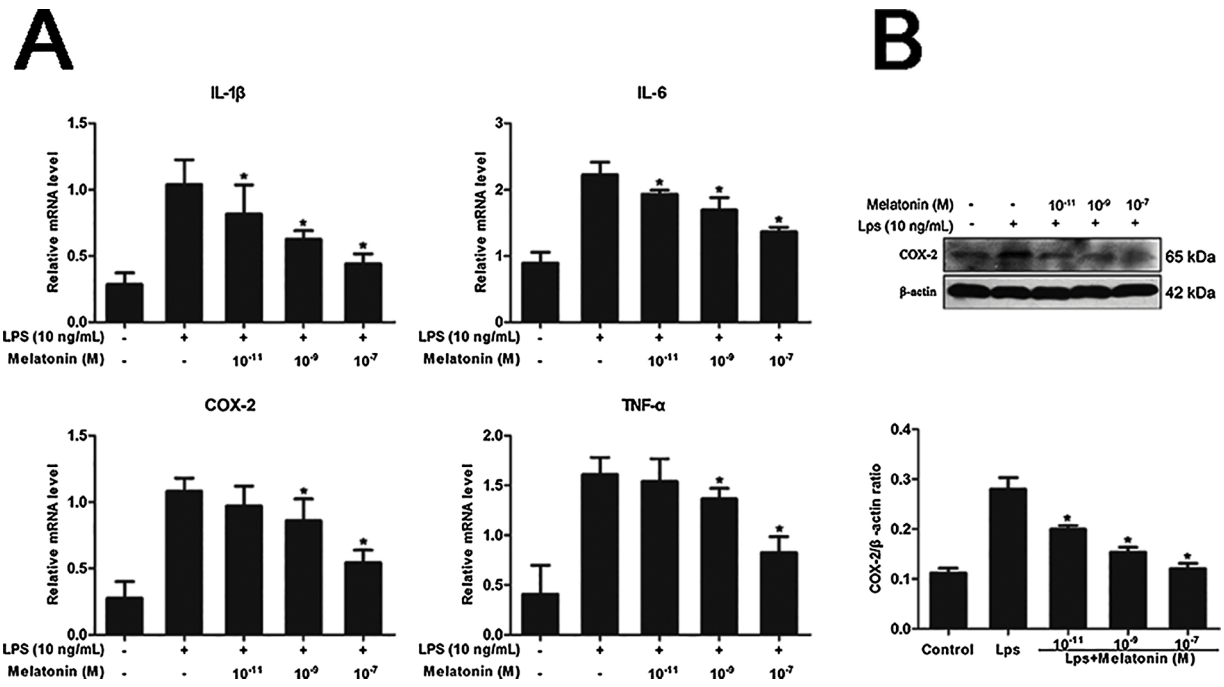

Fig. 1. Effect of melatonin on IL-1β, IL-6, COX-2, and TNF-α expression in LPS-induced sheep epididymal epithelial cells. (A) Relative mRNA levels of IL-1β, IL-6, COX-2, and TNF-α detected by detected by qRT-PCR; β-actin served as the internal reference gene. (B) Detection of COX-2 expression by western blotting; β-actin was used as the loading control. Data are presented as mean ± SEM (n = 3). *P < 0.05, melatonin-treated cells vs. untreated LPS-induced cells.

the inhibitory effect of melatonin on the expression of inflammatory cytokines IL-1β, IL-6, COX-2, TNF-α, TLR4, p-NF-κB-p65, and p65, and the inhibitory effect of the non-selective receptor antagonist luzindole was more obvious than that of 4P-PDOT. The anti-inflammatory effect of melatonin was shown to be principally exerted through MT2 [42]. In pancreatic tissue inflammation, endogenous melatonin decreases inflammatory cytokine production via MT2, thereby improving tissue resistance [27]. Our findings indicated that both MT1 and MT2 mediated the anti-inflammatory effects of melatonin; the fact that the non-specific receptor blocker luzindole had a greater effect than the specific receptor blocker may be attributable to the different species or sources of melatonin. In addition, aralkylamine N-acetyltransferase and hydroxyindole-O-methyltransferase, which are key enzymes in melatonin synthesis, are expressed in the testicles and cauda epididymis of male animals [43], indicating that sheep epididymis may spontaneously synthesize and secrete melatonin, which could have an autocrine autoimmune effect on the epididymitis. In this study, we found the physiological concentration of melatonin plays a part in inhibiting the inflammation of epididymal epithelial cells, but the inflammation of epididymal epithelial cells cannot be completely eliminated. Because melatonin has a very short half-life [44], melatonin concentration *in vitro* experimental models may have been much lower than that used *in vivo*. However, the pathogenesis of epididymitis is complex, and the effects of melatonin administration depend on the dose [45], time of application [46], and duration of application [47]; thus, treatment of epididymitis in large animals with this method has certain limitations. Repeated injections or continuous infusion may be necessary for melatonin to exert its full effect. Therefore, more clinical studies are needed

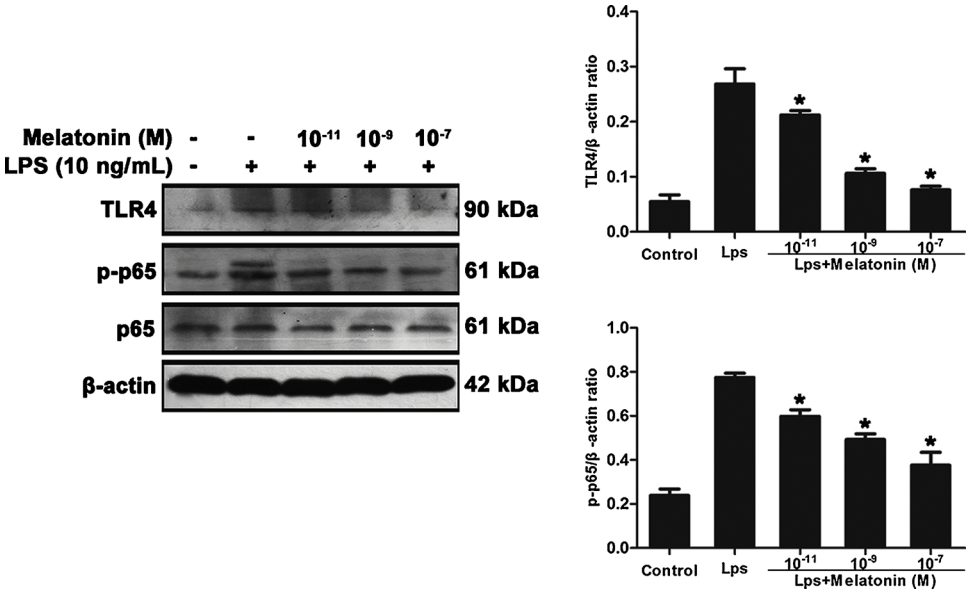

Fig. 2. Effect of melatonin on TLR4 expression and NF-κB activation in LPS-induced sheep epididymal epithelial cells. TLR4 protein level and NF-κB p65 phosphorylation were detected by western blotting; β-actin was used as the loading control. Data are presented as mean ± SEM (n = 3). *P < 0.05, melatonin-treated cells vs. untreated LPS-induced cells.

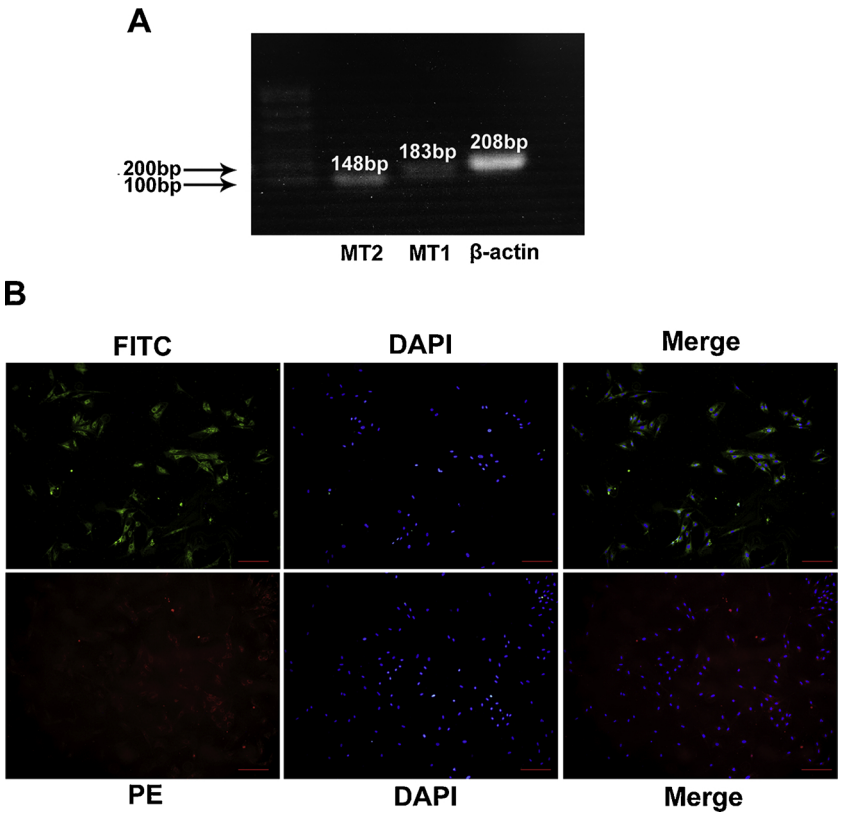

Fig. 3. MT1 and MT2 expression in cultured sheep epididymal epithelial cells. (A) MT1 and MT2 expression detected by qRT-PCR. (B) Detection of MT1 (FITC; green) and MT2 (PE; red) by immunofluorescence microscopy; nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Scale bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

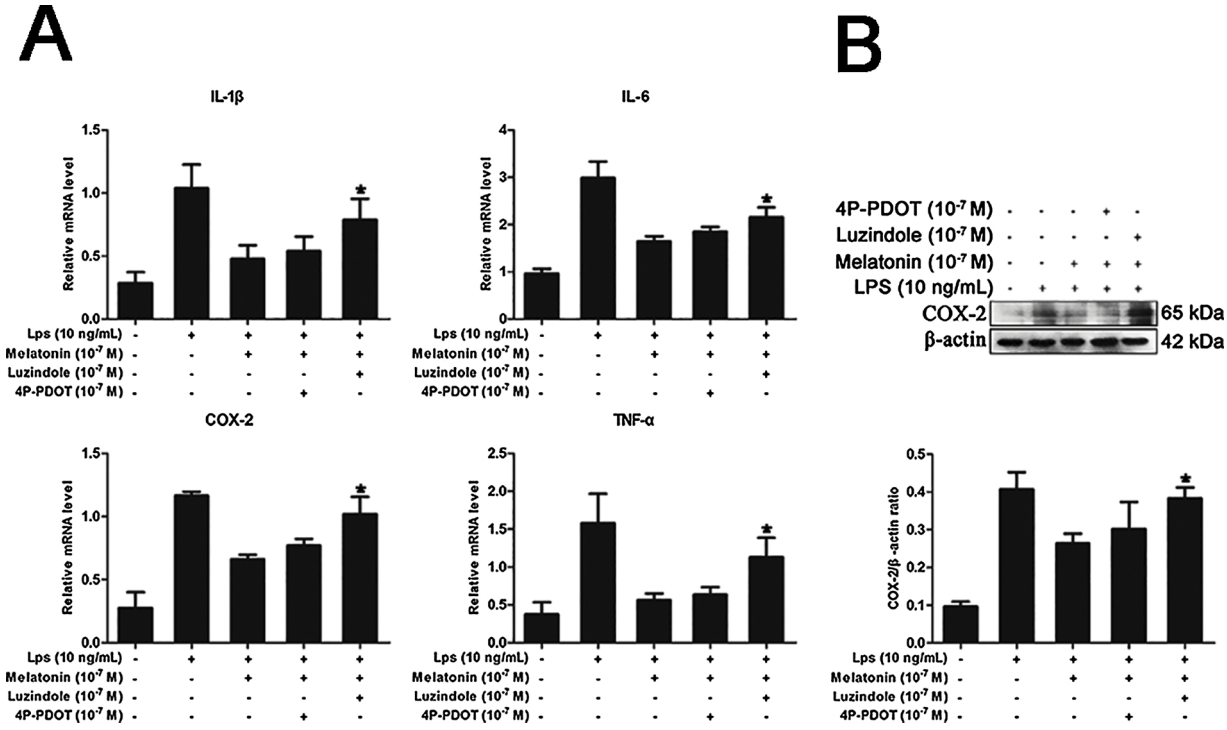

Fig. 4. Role of MT1 and MT2 on inhibition of LPS-induced inflammation by melatonin in sheep epididymal epithelial cells. (A) Relative mRNA levels of IL-1β, IL-6, COX-2, and TNF-α detected by qRT-PCR; β-actin served as the internal reference gene. (B) Detection of COX-2 expression by western blotting; β-actin was used as the loading control. Data are presented as mean ± SEM (n = 3). *P < 0.05, LPS + melatonin + luzindole- or 4P-PDOT-treated cells vs. LPS + melatonin-treated cells.

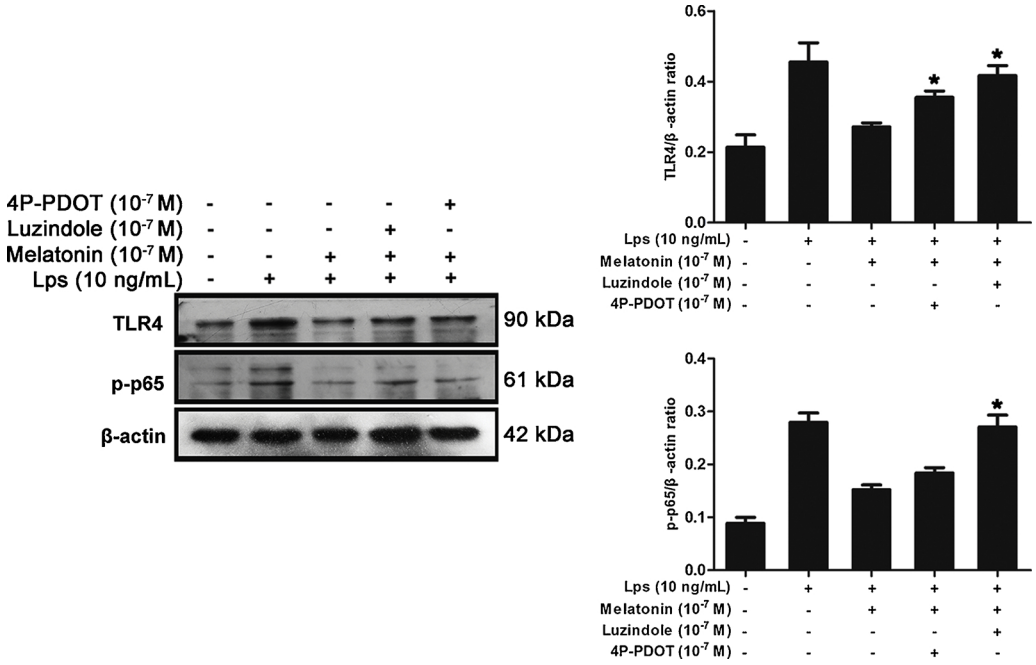

Fig. 5. Effects of MR antagonist luzindole and 4P-PDOT on inhibition of TLR4 and NF-κB signaling by melatonin in LPS-induced sheep epididymal epithelial cells. Cells were treated with luzindole and 4P-PDOT and TLR4 and NF-κB p65 levels were evaluated by western blotting; β-actin was used as the loading control. Data are presented as mean ± SEM (n = 3). *P < 0.05, LPS + melatonin + luzindole- or 4P-PDOT-treated cells vs. LPS + melatonin-treated cells.

to validate the benefits of melatonin, and to elucidate the specific mechanism of melatonin in sheep epididymitis.

5. Conclusion

In conclusion, our results indicate that melatonin has an anti-inflammatory effect on LPS-induced epididymitis that is exerted through MT1 and MT2. This activity inhibits LPS-induced TLR4 and NF-κB activation, thereby suppressing the production of pro-inflammatory factors. These results indicate that melatonin may be effective alternative to existing drugs for the treatment of sheep epididymitis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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