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RESEARCH ARTICLE

Melatonin-Mediated Regulation of Circadian Protein Cryptochrome Signaling in Suppressing Epididymo-Orchitis in Bactrian Camels

Shuai Ji[1], Mengwei Li[1], Jibao Hou[1], Yuting Fang[1], Chengning Wu[1], Yuan Gao[1] and Shuqin Zhao[2*]

[1]College of Life Science and Technology, Gansu Agricultural University, Lanzhou 730070, China; [2]Laboratory and base management center, Gansu Agricultural University, Lanzhou 730070, China
*Corresponding author: zhaosq@gsau.edu.cn

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A B S T R A C T

Epididymo-orchitis is a common disease in Bactrian camels that affects male fertility and animal husbandry development, but the regulatory mechanism of epididymo-orchitis is not clear. The present study was planned to focus on the anti-inflammatory effects of melatonin on its receptor (MT1/2) and explore the mechanism of regulation of inflammation by cryptochromes (Cry1/2) as an activating protein of MT1/2. An *in vitro* inflammation model was established using primary epithelial cells of the caput epididymis of six slaughtered Bactrian camels, together with an *in vivo* inflammation model of mice epididymo-orchitis was established to explore the role of MT1/2-Cry module in regulating inflammation. Subsequently, histological changes (H&E staining assay), protein expression (immunohistochemistry and Western blot assay), gene expression (RT-qPCR assay), cytokine levels (ELISA), and sperm malformation rates were assessed. The *in vitro* experimental results showed that melatonin relied on its receptor MT1/2 to inhibit the expression of inflammatory markers (*CD14*, *TLR4*, *MYD88*, N*F-κB*, *IL-6*, *IL-18*) by activating the TLR4/NF-κB signaling pathway. The performance of combination of Cry1/2 and melatonin was better than that of melatonin alone, and the anti-inflammatory effect of Cry was regulated by MT1/2. The results of *in vivo* inflammatory model experiments showed that melatonin improved the pathological damage of the testis and epididymis, and upregulated the expression of genes associated with germ cell structure and cell adhesion-related genes (*Nectin3*, *PRM1*, *TH2B*). Through *Cry* gene intervention and melatonin combination therapy, it was found that Cry1 and melatonin had a synergistic effect, and their combination can more effectively inhibit inflammatory responses and improve reproductive-related parameters. This study provides new potential targets and theoretical basis for the treatment of epididymo-orchitis in Bactrian camels.

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INTRODUCTION

Clock genes are crucial for maintaining physiological rhythms and cellular functions in the body. The main clock genes, including *Clock, Bmal1, Per1/2/3, Cry1/2, Rev-erbα* and *Rorα* (Walker *et al*., 2020), serve as the molecular basis of circadian rhythms, and form autonomous transcription-translation feedback loops to drive gene expression that influences oxidative stress, immune responses and tissue repair (Wang *et al*., 2021a; Wang *et al*., 2023). In recent years, close association between the circadian rhythm system and immune responses has become a key focus in inflammation research. Among all the proteins encoded by circadian clock genes, cryptochrome (Cry1 and Cry2) proteins are the core components and transcription factors of the biological clock. Cry1 and Cry2 proteins not only participate in regulating the circadian rhythm but also directly control the expression level of pro-inflammatory mediators (Lamia *et al*., 2011; Qin and Deng, 2015). Previous studies have shown that in *Cry1/2* double knockout mice, *tumor necrosis factor-α* (*TNF-α*), *interleukin-6* (*IL-6*) and *interleukin-1β* (*IL-1β*) are abnormally overexpressed in the spleen and macrophages, while the nuclear factor kappa B (NF-κB) and protein kinase A (PKA) signaling pathways are continuously

activated, thereby aggravating inflammatory damage (Narasimamurthy *et al*., 2012). This pro-inflammatory phenotype is of great clinical significance. For instance, in experimental arthritis mouse models, the condition of mice lacking *Cry1* and *Cry2* proteins would worsen. However, the increased expression of *Cry1* from the outside directly inhibits the promoter activity of pro-inflammatory factors such as TNF-α and reduces the level of serum cytokines (Hashiramoto *et al*., 2010). In the atherosclerosis model, *Cry1* highlights its anti-inflammatory and vascular protective effects by directly inhibiting the expression of Toll-like receptor 2/4 (TLR2/TLR4) and NF-κB p65 subunit (p65) phosphorylation, reducing the expression of vascular adhesion molecules, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1; Yang *et al*., 2015). In addition, *Cry1* and *Cry2* have functional differences. When synovial cells are stimulated with TNF-α, the expression of *Cry1* is significantly upregulated, while that of *Cry2* remains unchanged (Yoshida *et al*., 2013), suggesting that they may play different roles in the inflammatory response. Moreover, there is a complex association between biological rhythm genes and inflammation. Biological clock genes can regulate the expression of inflammation related genes (Keller *et al*., 2009; Gibbs *et al*., 2012), and inflammatory signals can also affect the expression of biological clock genes, thereby forming a bidirectional regulatory network (Orozco-Solis and Aguilar-Arnal, 2020; Shen *et al*., 2021; Cox *et al*., 2022).

Regarding the anti-inflammatory mechanism of *Cry*, studies have found that *Cry1* binds to adenylate cyclase to inhibit the production of cAMP, thereby blocking PKA-mediated phosphorylation of NF-κB p65 and forming the "Cry-cAMP-PKA-NF-κB" inhibition axis (Narasimamurthy *et al*., 2012). In addition, defense response of the host has also been linked to circadian rhythm events. Transcription factors within the cell nucleus, such as Cry, coordinate melatonin from both the pineal gland and non-pineal gland sources (such as colon, retina), which jointly participate in the initiation and regression of innate immune responses (Vriend and Reiter, 2015; Blancas-Velazquez *et al*., 2023). Non-pineal melatonin is widely distributed in the reproductive system, while testicles and epididymis tissues can synthesize melatonin autonomously through self-expressed melatonin synthase, rather than relying solely on systemic melatonin secreted by the pineal gland (Gonzalez-Arto *et al*., 2016). Melatonin exerts its effect through G protein-coupled membrane receptors. In humans and mammals, melatonin has two main receptors, including melatonin receptor 1 (MT1) and melatonin receptor 2 (MT2). Melatonin exerts anti-inflammatory activity by inhibiting the cAMP–PKA–NF-κB pathway through MT1 and MT2. For instance, in the lipopolysaccharide (LPS)-induced orchitis model of sheep, melatonin remarkably downregulated the expression levels of TNF-α, IL-1β and IL-6, while inhibiting TLR4 protein expression and NF-κB nuclear translocation (Deng *et al*., 2020). Similarly, in the LPS (6mg/kg)-induced sepsis-induced myocardial injury model in mice, melatonin alleviated myocarditis by activating MAPK-dependent autophagy, while serum IL-6 and TNF-α also decreased (Di *et al*., 2020). Mechanism studies further indicated that melatonin could directly block the activation of the myeloid differentiation primary response protein 88 (MYD88) adapter protein downstream of TLR4, thereby inhibiting the degradation of IκBα and the phosphorylation of p65, ultimately suppressing the transcriptional activity of *NF-κB* (Xu *et al*., 2018; Chamanara *et al.,* 2019). Importantly, MT1/2 plays a basic role in its anti-inflammatory effect. According to Xi *et al*. (2021), knockdown of the *MT1* receptor in intestinal epithelial cell lines and organoid models weakens the inhibitory effect of melatonin on NF-κB and IL-6/IL-8. In the LPS-induced mouse colitis model, melatonin acts through the MT2 receptor, inhibiting the abnormal upregulation of methyltransferase-like 3 (METTL3), reducing m^6A methylation, suppressing M1 macrophage polarization and promoting M2 polarization, thereby reducing inflammation (Li *et al*., 2024).

Although both melatonin and *Cry1/2* act on the TLR4/NF-κB pathway, whether there is a synergistic effect between them has not been systematically clarified yet. Multiple lines of evidence suggest that circadian mechanisms may be critical mediators of anti-inflammatory actions of melatonin. It may regulate circadian rhythms through dual mechanisms: directly upregulating *Cry2* expression and indirectly activating the MT1–PITX1 axis, thereby suppressing inflammation (Vriend and Reiter, 2015; Huang *et al*., 2018; Guo *et al*., 2024). For example, in a rheumatoid arthritis–associated pulmonary fibrosis model, melatonin treatment markedly increased expression of *Cry2* and *Per2* in alveolar epithelial cells, increased E-cadherin expression, and reduced macrophage infiltration as well as epithelial–mesenchymal transition (Huo *et al*., 2025). Our previous work demonstrated that overexpression of *MT1/2* inhibited cAMP production and significantly increased *Cry1/2* expression, while overexpression of *Cry1/2* likewise inhibited cAMP production and enhanced MT1/2 expression (Zhao *et al*., 2021). Dual-luciferase reporter assays further revealed that *Cry* regulates *MT1* expression at the transcriptional level (Zhao *et al*., 2025). Therefore, elucidating the mechanisms by which MT-mediated Cry activity suppresses inflammation is of great importance for identifying novel therapeutic targets and strategies for inflammatory diseases.

The Bactrian camel is an important livestock resource in the arid desert regions of northwestern China, and its reproductive activity exhibits pronounced seasonality, with estrus in females typically occurring in winter, when males are sexually active. This seasonal reproductive pattern in camelids is generally considered to be associated with changes in photoperiod and harsh environmental conditions (Ali *et al*., 2024). Male fertility is often compromised by reproductive tract inflammatory diseases such as epididymo-orchitis (Hassaneen *et al*., 2024), which severely affect animal health and reproduction. Current anti-inflammatory treatments mainly rely on glucocorticoids and biologics, but long-term use is prone to cause drug resistance and obvious side effects (Boyman *et al*., 2014). Since both melatonin and Cry are involved in regulating the inflammatory process, speculating that the combination of the two may

have a better synergistic effect in alleviating epididymo-orchitis in Bactrian camels.

Therefore, an *in vitro* inflammation model was constructed using the caput epididymis epithelial cells of Bactrian camels, and an *in vivo* model of epididymo-orchitis in mice was established simultaneously in this study, for systematically exploring the synergistic effect of melatonin and Cry in inhibiting inflammation and its molecular mechanism. The aim of the present study was to elucidate the possible role of melatonin–MT1/2–Cry axis in the regulation of male reproductive tract inflammation.

MATERIALS AND METHODS

Experimental animals: Six adult male Alxa Bactrian camels were randomly selected from a slaughterhouse in Zhangye, Gansu Province, China. After slaughter, testis and epididymis tissues were collected. Each tissue sample was divided into two parts: one part was stored in phosphate-buffered saline (PBS) with 5% penicillin–streptomycin and the other part was fixed in 4% paraformaldehyde for hematoxylin–eosin (H&E) staining and immunohistochemical analysis for Cry1, MT1 and MT2. The classification of normal versus inflamed tissues was based on established histopathological criteria under H&E staining, including inflammatory cell infiltration, disruption of tissue architecture, and interstitial edema, as described previously (Ali *et al.*, 2024).

Primary cell isolation and culture: The caput epididymis tissues of Bactrian camels were washed with PBS and dissected into small pieces. The fragments were digested with 1.0g/mL collagenase IV (Sigma, USA), and the isolated cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). The cells were seeded into 6-well plates at a density of 0.5×10^6 cells/cm². After 24h, non-adherent cells were removed, and the adherent cells were purified through gradient digestion. Epithelial cells were identified by immunofluorescence using cytokeratin 18 (Luan *et al*., 2021).

Histopathological examination: Testicular and epididymal tissues taken from Bactrian camels were fixed in 4% paraformaldehyde overnight, dehydrated, cleared in xylene, embedded in paraffin, and sectioned (5 μm). Sections were stained with hematoxylin and eosin (H&E) staining using standard technique (Ali *et al*., 2024). For immunohistochemistry, primary antibodies against Cry1, MT1, MT2, CD14 and TLR4 were incubated overnight at 4°C. After washing, sections were incubated with HRP-conjugated goat anti-rabbit IgG, then stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB), using a commercial kit (Solarbio, China), and finally counterstained with hematoxylin. Detailed antibody information is provided in Table 1. Images were captured using a DM3000 optical microscope (Leica, Germany) and a confocal microscope (LSM 780, Carl Zeiss, Germany).

Cell inflammation model: Primary epithelial cells from the caput epididymis of camels were seeded into 35 mm dishes. Upon reaching 80–90% confluence, cells were treated with 200 ng/mL LPS for 2 h, followed by medium replacement and incubation for 6, 24, or 48 h. In the melatonin (Mel) group, cells were treated with 10^{-6}M melatonin. The LPS+Mel group received melatonin after 2 h of LPS exposure. The LPS+luzindole+Mel group involved pretreatment with the melatonin receptor antagonist luzindole (10 μM) before melatonin administration. *Cry1/2* overexpression was achieved by transfection with plasmid pEGFP-*Cry1/2* for 24 h before treatment with LPS or melatonin.

Table 1: Antibodies used in Immunohistochemistry (IHC)

Antibody	Catalogue No.	Company	Dilution	Country
Cryptochrome 1 Rabbit pAb	#bs-11441R	Bioss	1:300	China
MT1 Rabbit pAb	#bs-0027R	Bioss	1:300	China
MT2 Rabbit pAb	#bs-0963R	Bioss	1:300	China
CD14 Rabbit pAb	#bs-1192R	Bioss	1:300	China
TLR4 Rabbit pAb	#bs-20594R	Bioss	1:300	China
HRP Goat Anti-Rabbit IgG	#RS0002	Immunoway	1:100	USA

After treatment, culture supernatants were collected to quantify IL-6 and IL-18 concentrations using ELISA kits (#JL22488 and #JL16235, respectively; Jianglai Biology, China). Cells were harvested separately and then RNA and proteins were extracted for subsequent molecular experimental analysis.

Quantitative Reverse Transcription PCR (RT-qPCR): Total RNA was extracted using TRIzol reagent (Cowin Biotech, China), and then cDNA was obtained by reverse transcription using RevertAid RT kit (Thermo Fisher, USA). Then, real-time quantitative PCR was performed using the UltraSYBR mixed PCR kit (Cowin Biotech, China). The expressions of genes such as *GAPDH*, *CD14*, *TLR4*, *MYD88*, *TNF-α*, *IL-6*, *IL-18*, *NF-κB*, *PRM1* (protamine 1*)*, *Nectin3* (nectin cell adhesion molecule 3) and *TH2B* (testis-specific histone H2B variant) were detected. Primer sequences were designed and validated using Primer 6 and BLAST (NCBI), as shown in Tables 2 and 3. All experiments were performed in triplicate. Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Western Blotting: Proteins were extracted from primary epididymal epithelial cells of Bactrian camels using RIPA lysis buffer (Solarbio, China) and quantified using a BCA protein assay kit (Solarbio, China). Equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were blocked and incubated overnight with primary antibodies: CD14, MYD88, Nectin3, and β-Tubulin (all from Immunoway, USA). After washing, membranes were incubated with HRP-conjugated goat anti-rabbit IgG (Immunoway, USA) and visualized using ECL substrate (Vazyme, China). Full antibody details are shown in Table 4.

Mice epididymo-orchitis model establishment and drug treatment: A total of 36 clinically healthy, male mice, aged 8 weeks and weighing 25-30 g, were used in this study. These mice were housed under standard

conditions (22–25°C, 12 h light/dark cycle) with free access to standard mice feed and water. These mice were used to establish an experimental model of epididymo-orchitis, as described below.

After an acclimatization period of 2 weeks, experimental mice were randomly assigned into six groups (6 mice/group): normal control, inflammation model (LPS), positive control (LPS+Dexamethasone: LPS+DXM), experimental group I (LPS+Mel), experimental group II (Overexpression *Cry1*+LPS), and experimental group III (Overexpression *Cry1*+LPS+Mel). Mice in the normal control group received intraperitoneal injections of saline, while all other groups were injected intraperitoneally with LPS at 2 mg/kg, once every two days for 6 days (three injections in total) to establish the epididymo-orchitis model (Singh *et al*., 2024). Following model induction, the LPS+DXM group (positive control) received DXM (10 mg/kg/day) by gavage for 5 days (Al-Harbi *et al*., 2016), while experimental group I received melatonin (40 mg/kg/day) by gavage for 5 days (Wang *et al*., 2021b). Experimental groups II and III received subcutaneous scrotal injections of plasmid pEGFP-*Cry1* (40 μg/mouse), as suggested previously (Michaelis *et al*., 2014; Qin and Deng, 2015), followed by LPS injection or melatonin gavage.

After treatments, mice were slaughtered after anesthesia, and testis and epididymis were collected for histological, immunohistochemical, RT-qPCR, and Western blot analyses. The specific operation method was the same as that described for the Bactrian camels. The antibodies used in immunohistochemistry are shown in Table 1, the primer sequences of RT-qPCR are summarized in Table 3, and the antibody information used in Western blot is listed in Table 4.

Table 2: Primer sequences of Bactrian camel for RT-qPCR

Primer name	Accession number	Forward sequence, 5′to 3′	Reverse sequence, 5′to 3′	Product length (bp)
MT1	XM_010953355	GTGGTGGTGTTCCATTTCATAGTT	GGGCTTTAGTTTCGGTTTGTTG	177
MT2	XM_010973367	GATCCCAGAGGGGTTGTTTG	TTCCAGAGGGCAGAGACGA	146
Cry1	XM_010970474	CACTGGTTCCGAAAGGGGCTC	CTGAAGCAAAAATCGCCACCT	145
Cry2	XM_010964628	CACTGGTTCCGCAAGGGATTG	CCAAGGGTCGAGGATGTAAA	134
CD14	XM_010971752	CAAACTCAGCGTGCTTGATCTC	GATTCCCATCCAGTGTCAGGTT	98
MYD88	XM_010945839	GAGAAGAGGTTGGCTAGGTG	GAGAGAGGCTGAGTGCAAACT	121
TLR4	XM_010957186	AGCGGAAACCACCTCATGTTAT	AGTGGAGGGGGTATCTGAATCA	164
NF-KB	XM_074343171	AAGATGCCCCACGATTACGG	TTCTCACACAGACAGTGCGG	119
IL-18	XM_010968922	CCTGGAATCAGATTACTTTGGC	AGGCATATCCTCAAACACGGG	116
IL-6	XM_010968610	GAAACACTGAGGCTGTGCAAAT	TGATTGGGTTAGGGGTGCTTAC	99
TNF-α	NM_001319779	TGTTCCTCACTCACACCATCAG	AAGTCGATCATCCTTCTCCAGC	176
GAPDH	XM_010957730	CTGGTGCTGAGTACGTTGTGGAG	AGGAGGCGTTGCTGACAATCTTG	96

Table 3: Primer sequences of mouse for RT-qPCR

Primer name	Accession number	Forward sequence, 5′to 3′	Reverse sequence, 5′to 3′	Product length (bp)
MT1	NM_008639	GTTTACCCTTATCCCTTGGTGC	GCGCTGACTTGACAGTGTAGAT	80
MT2	NM_145712	GCCTTGGCTGACTTGGTGATA	GACCCAACCGTCACGGATAA	75
Cry1	NM_007771	AGGTGGCGATTTTTGCTTCA	CGAATCACAAACAGACGAGA	80
Cry2	NM_009963	GTGCACTGGTTCCGCAAAG	ATCGGTTGATGCCCACAGAC	139
CD14	NM_009841	ACTTCTCAGATCCGAAGCCAG	CCGCCGTACAATTCCACAT	76
TLR4	XM_036163964	ATGGCATGGCTTACACCACC	GAGGCCAATTTTGTCTCCACA	129
MYD88	NM_010851	TCATGTTCTCCATACCCTTGGT	AAACTGCGAGTGGGGTCAG	175
NF-κB	NM_001410442	ATGGCAGACGATGATCCCTAC	TGTTGACAGTGGTATTTCTGGTG	111
IL-1β	NM_008361	CACTACAGGCTCCGAGATGAACAAC	TGTCGTTGCTTGGTTCTCCTTGTAC	145
TNF-α	NM_013693	GGACTAGCCAGGAGGGAGAACAG	GCCAGTGAGTGAAAGGGACAGAAC	103
PRM1	NM_013637	GCCCACAAAATTCCACCTGC	AGCATCGGTATCTGGCCATG	108
TH2B	NM_175663	CCTGGCGCATTACAACAAGC	TGGAGCTGGTGTACTTGGTG	137
Nectin3	NM_021496	GTTGAACCCACAGTGAGCCT	TGCTACACAAACGGCTGCTA	84
GAPDH	NM_008084	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA	95

Table 4: Antibodies used in Western-blotting experiment

Antibody	Catalogue No.	Company	Dilution	Country
CD14 Rabbit mAb	#YM8259	Immunoway	1:4000	USA
MYD88 Rabbit pAb	#YT2928	Immunoway	1:1200	USA
Nectin3 Rabbit pAb	#YT5261	Immunoway	1:1200	USA
β-Tubulin Rabbit mAb	#YM8332	Immunoway	1:5000	USA

Hematological analysis: After drug treatments, peripheral blood from mice was collected under anesthesia to minimize animal pain and stress (Li *et al*., 2025). The total number of leukocytes, as well as the neutrophils percentage (NEU%), was determined using a fully automated hematology analyzer (URIT-2900Vet Plus, URIT, China).

Testis index and sperm abnormality rate: After completion of all treatments, mice were fasted for 10 h, anesthetized, and then sacrificed. Testes were excised, cleaned of fat and connective tissue, blotted dry, and their weight was recorded for the calculation of the testis index, as shown below:

$$\text{Testicular index} = \frac{W_{\text{testis}}}{W_{\text{body}}} \quad (\text{mg/g})$$

After sacrifice, testicular and epididymal tissues were also collected for subsequent histopathological and immunohistochemical analyses using the same procedures as described for Bactrian camels.

Mice epididymal sperm were collected by mincing the epididymis in prewarmed saline, sperm smears were prepared, air dried, stained with eosin stain, and examined under a light microscope (200× and 500× magnification) for sperm morphology. At least 200 sperm were evaluated per sample, and sperm with head, mid-piece, or tail abnormalities were classified as abnormal. The sperm abnormality rate was calculated using the following formula:

$$\text{Sperm abnormality rate } (\%) = \left(\frac{\text{abnormal sperm count}}{\text{total sperm count}}\right) \times 100$$

Statistical analysis: Data were analyzed using SPSS 22.0 and GraphPad Prism 8.3.0. Values are expressed as mean ± standard error (SE). One-way analysis of variance was used for statistical differences between groups, followed by Tukey's multiple comparisons post-hoc test. The $P<0.05$ indicated statistically significant difference, while $P<0.01$ indicated highly significant difference.

RESULTS

Histopathological observations of epididymo-orchitis in Bactrian camels: To characterize the histopathological features of epididymo-orchitis in Bactrian camels, paired testicular and epididymal tissues were examined from six randomly selected Bactrian camels. Inflammatory cell infiltration was observed in four of the six cases, indicating that inflammatory lesions were prevalent in the reproductive organs of Bactrian camels.

Histopathological examination revealed that seminiferous tubules in normal testes were tightly arranged with normal epithelial thickness and orderly distribution of germ cell layers (Fig. 1). In contrast, inflamed testes exhibited enlarged intertubular spaces, thinning of the seminiferous epithelium, reduced and disorganized germ cell layers, and detachment of spermatogenic cells into the lumen, where abundant cellular debris and cytoplasmic remnants were observed. Interstitial regions appeared loose and edematous due to increased vascular permeability. In the normal epididymis, the ducts were filled with mature sperm, and the epithelial structure was intact and neatly arranged. In the epididymis with inflammation, the sperm density in the lumen was reduced or even completely disappeared, and replaced by shed epithelial cells and cell debris. The epithelial layer showed collapse, thinning and structural disorder, and at the same time, the plasma area was expanded and was accompanied by edema.

The expression and localization of melatonin receptors (MT1/MT2) and Cry proteins showed that in normal testicular and epididymal tissues, Cry1, MT1 and MT2 were all strongly expressed, mainly located in germ cells, supporting cells and epididymal epithelial cells, with uniform staining and obvious signals (Fig. 1). In the inflamed organs, the expressions of Cry1 and MT1 appeared higher, the staining was deeper, and enhanced positive signal areas could be seen locally. In contrast, the expression of MT2 in inflamed organs was comparable to that in organs of the control group.

Anti-inflammatory effect of melatonin: Primary epithelial cells isolated from the caput epididymis of Bactrian camels were triangular in shape, evenly distributed and showed good growth condition. Immunofluorescence staining showed that over 99% of the cells expressed the epithelial cell marker cytokeratin-18, confirming the successful purification (Fig. 2A). The results of the in vitro inflammation model (Fig. 2B) showed that at the early stage (6 h), treatment with melatonin alone led to higher expression levels of *CD14*, *TLR4*, *MYD88*, *NF-κB* and *IL-6* compared with the control group. By 24 h, the expression of these markers showed a decreasing trend, with several inflammation-related markers approaching control levels, but *IL-18*

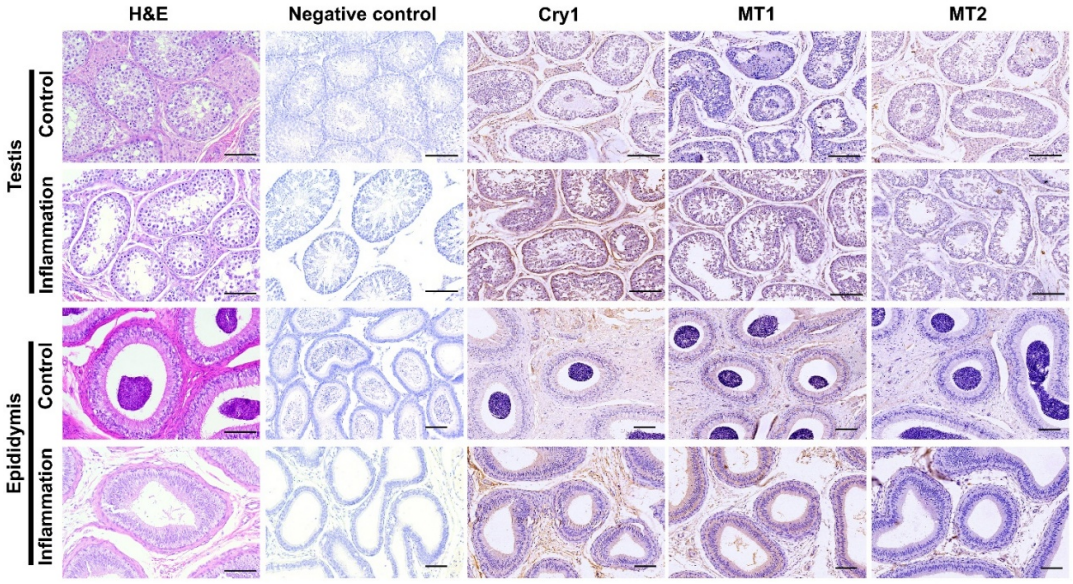

Fig. 1: Hematoxylin and eosin (H&E) staining and immunohistochemical analysis of testes and epididymides in Bactrian camels under normal and inflammatory conditions. Each row displays H&E, negative control, Cry1, MT1, and MT2 staining of the testis and epididymis; each column shows control testis, inflamed testis, control epididymis, and inflamed epididymis. Scale bar=100μm.

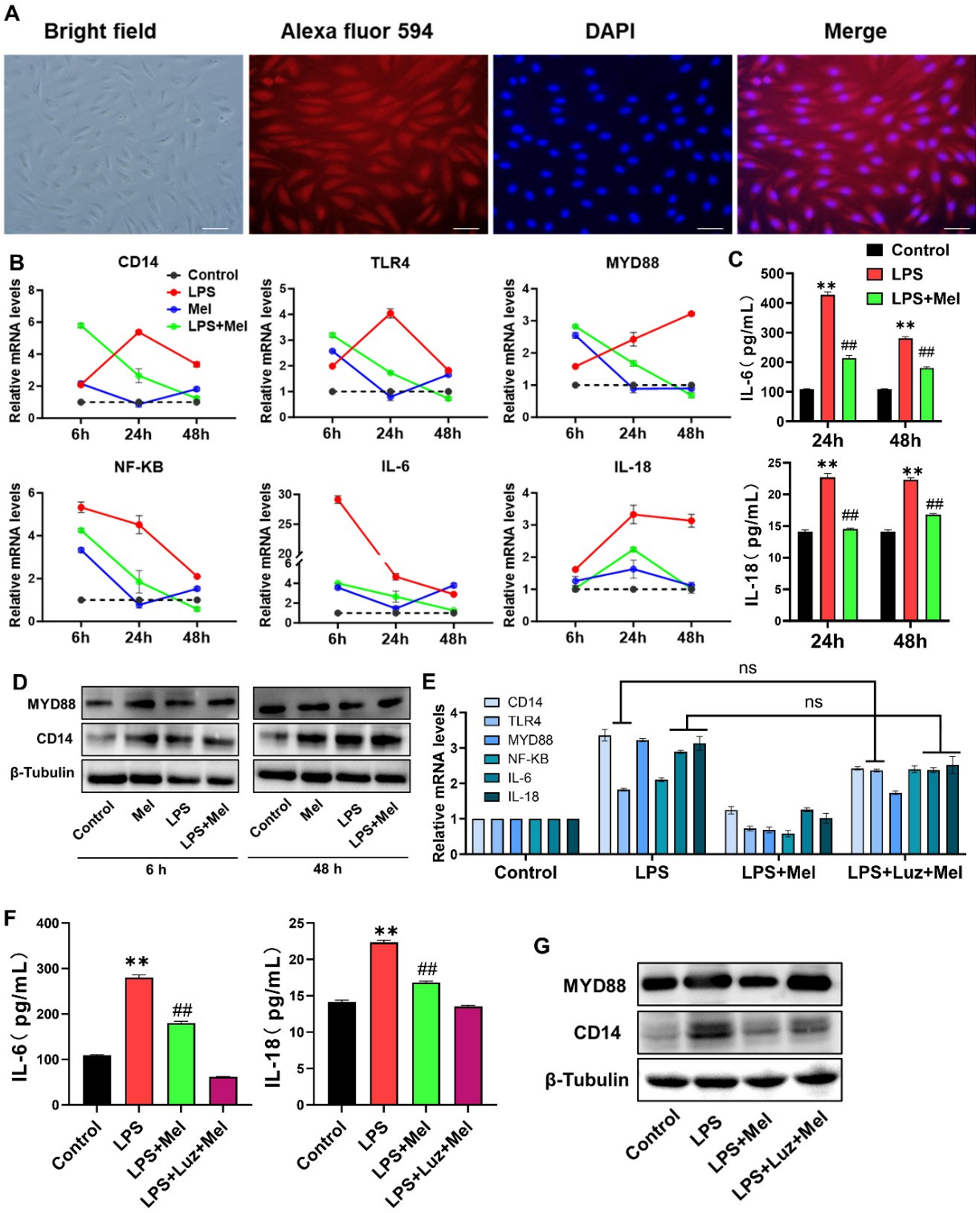

Fig. 2: The anti-inflammatory effects of melatonin (Mel) on caput epididymis epithelial cells of Bactrian camels. (A): Immunofluorescence identification of primary caput epididymal epithelial cells of Bactrian camel. Scale bar=20μm. (B): The mRNA expression levels of inflammation-related genes (*CD14, TLR4, MYD88, NF-κB, IL-6, IL-18*) in control and treatment groups at 6, 24, and 48h. (C): Secretion levels of IL-6 and IL-18 detected by ELISA in control, LPS and LPS+Mel groups at 24 and 48 h. (D): Western blot analysis of CD14 and MYD88 protein expression in control and treatment groups at 6 and 48 h; Tubulin served as a loading control. (E): The mRNA expression changes of inflammation-related genes under receptor inhibition in control and treatment groups. (F): Secretion levels of IL-6 and IL-18 showing receptor-dependent variations in control and treatment groups. (G): Western blot analysis of CD14 and MYD88 protein expression under receptor-dependent conditions in control and treatment groups; Tubulin served as a loading control. "Luz" is for the abbreviation of "Luzindole". Compared with control group: *P<0.05, **P<0.01; Compared with LPS group: #P<0.05, ##P<0.01.

remained highly expressed. At 48h, the expression of *IL-18* also dropped to the baseline level, and TLR4 and *IL-6* expressions increased again. Following LPS stimulation, the expression of *NF-κB* and *IL-6* increased and reached their peak values at 6h. The expression of *CD14, TLR4* and *IL-18* reached their peaks at 24 h, while MYD88 peaked at 48h. In the LPS+Mel combined treatment group, the expression of inflammatory genes, except IL-18, was suppressed at 24 h and their expression was restored to normal at 48 h. The ELISA results of *IL-6* and *IL-18* secretion (Fig. 2C) were consistent with the findings of RT-qPCR, and the expression trends of CD14 and MYD88 proteins (Fig. 2D) were also in agreement with the RT-qPCR results, jointly confirming that melatonin has a time-dependent bidirectional effect-presenting as pro-inflammatory in the early stage and then exerting strong anti-inflammatory activity.

The anti-inflammatory effect of melatonin is achieved through its receptors: To determine whether melatonin exerts its anti-inflammatory effect via its receptors, caput epididymis epithelial cells of Bactrian camels stimulated with LPS were first treated with the receptor antagonist luzindole, followed by melatonin administration. Results from real-time quantitative PCR analysis (Fig. 2E) showed that melatonin inhibited LPS-induced upregulation of inflammatory genes, yet this inhibitory effect was attenuated in the presence of luzindole. This confirmed that anti-inflammatory effect of melatonin is dependent on the receptor signaling pathway. Consistent results were also observed at the protein level. ELISA assays demonstrated that melatonin significantly suppressed LPS-induced IL-6 and IL-18 secretion ($P<0.01$; Fig. 2F). Western blotting confirmed that melatonin inhibited LPS-induced upregulation of CD14 and MYD88 proteins, whereas this inhibition was diminished by receptor blockade (Fig. 2G).

***Cry* activation of *MT1/2* enhances the anti-inflammatory effects in Bactrian camel epididymal epithelial cells:** As shown in Fig. 3A, in Bactrian camel epididymal epithelial cells, overexpression *Cry1* resulted in upregulation of *MT1/2* expression, whereas overexpression *Cry2* suppressed *MT1* while increasing *MT2* expression. The results also showed that overexpression of either *Cry1* (Fig. 3B) or *Cry2* (Fig. 3C) significantly attenuated ($P < 0.01$) LPS-induced inflammatory gene expression (*CD14*, *TLR4*, *MYD88*, *NF-κB, IL-6*, and *IL-18*). Moreover, overexpression of *Cry2* combined with melatonin treatment suppressed inflammatory cytokines more effectively than *Cry1* plus melatonin, suggesting that *Cry2* may exert stronger synergistic effects with melatonin. Notably, overexpression of *Cry1* combined with melatonin under luzindole blockade showed partial loss of anti-inflammatory activity, indicating MT1/2 dependence. Likewise, the combined effect of overexpression of *Cry2* and melatonin also required MT1/2 receptors, which is consistent with receptor-mediated mode of action of melatonin. ELISA assays verified that overexpression of *Cry1* (Fig. 3D) or *Cry2* (Fig. 3E) led to decreased secretion of IL-6 and IL-18, with the combination of *Cry2* and melatonin demonstrating better efficacy. Further support for these findings came from Western blot analysis of CD14 and MYD88 protein expression (Fig. 3F).

Effects of melatonin on inflammation and integrity of reproductive tissues: Hematological tests in mice showed that LPS caused a significant increase ($P<0.01$) in the percentage of neutrophils compared to controls (Fig. 4A), with no effect on the total number of leukocytes (Fig. 4B). Melatonin treatment significantly reduced neutrophils ($57.96 \pm 1.82\%$, $P<0.01$), approaching control values ($46.90 \pm 3.39\%$) and outperforming DXM ($67.04 \pm 1.23\%$; Fig. 4A).

In terms of reproductive outcomes, LPS significantly increased ($P<0.01$) the testicular index compared to controls (Fig. 4C). In melatonin treated group, testicular index significantly decreased compared to LPS group ($P<0.05$), but differed non-significantly from control group. Macroscopic examination showed that testes from the LPS group exhibited obvious swelling, congestion of capillaries, whereas testes from the LPS+Melatonin group displayed appearance close to those of the control group. Representative sperm smears revealed that LPS exposure induced marked morphological abnormalities, which were significantly alleviated after melatonin treatment. Correspondingly, LPS significantly increased ($P<0.01$) the sperm malformation rate, reaching $44.08 \pm 16.58\%$ in the LPS group, compared to controls (Fig. 4D). After using Melatonin, this sperm abnormality rate was reduced ($P<0.01$), with the deformity rate decreased to $17.83 \pm 2.05\%$. Meanwhile, the testicular index, which was 3.99 ± 0.07mg/g in the LPS group, decreased to 3.52 ± 0.08mg/g following melatonin administration ($P<0.05$). All these indicators were close to the normal level, and the effect was better than that of DXM. Histological analysis also confirmed that melatonin significantly alleviated the damage to the testicles and epididymis, maintained the intact structure of the seminiferous tubules, reduced vacuolation, restored the arrangement of the epididymal epithelium, and brought the sperm and secretions in the lumen back to normal levels (Fig. 4E). Immunohistochemistry demonstrated that melatonin suppressed LPS-induced overexpression of CD14 and TLR4 (Fig. 5A).

Melatonin modulates inflammatory signaling and restores gene expression: Under different treatment conditions, the expression of inflammation-related genes associated with TLR4/NF-κB pathway and genes related to germ cell structure-related and cell adhesion was examined in mouse testes and epididymides. The results showed that LPS significantly activated the TLR4/NF-κB pathway, with *CD14, TLR4, TNF-α* and *IL-1β* mainly increased ($P<0.01$) in the testis (Fig. 5B), and *CD14* and *TNF-α* upregulated ($P<0.05$) in the epididymis (Fig. 5C) than the control group. Melatonin reversed these changes, restoring expression near to control levels, and this trend was further supported by protein expression analysis of CD14 and MYD88 in the testis (Fig. 5D). Additionally, LPS significantly downregulated *Nectin3*, *PRM1, and TH2B* expression than the controls ($P<0.01$) in the epididymis (Fig. 5F), with little effect in testes (Fig. 5E). Melatonin treatment significantly restored the expression

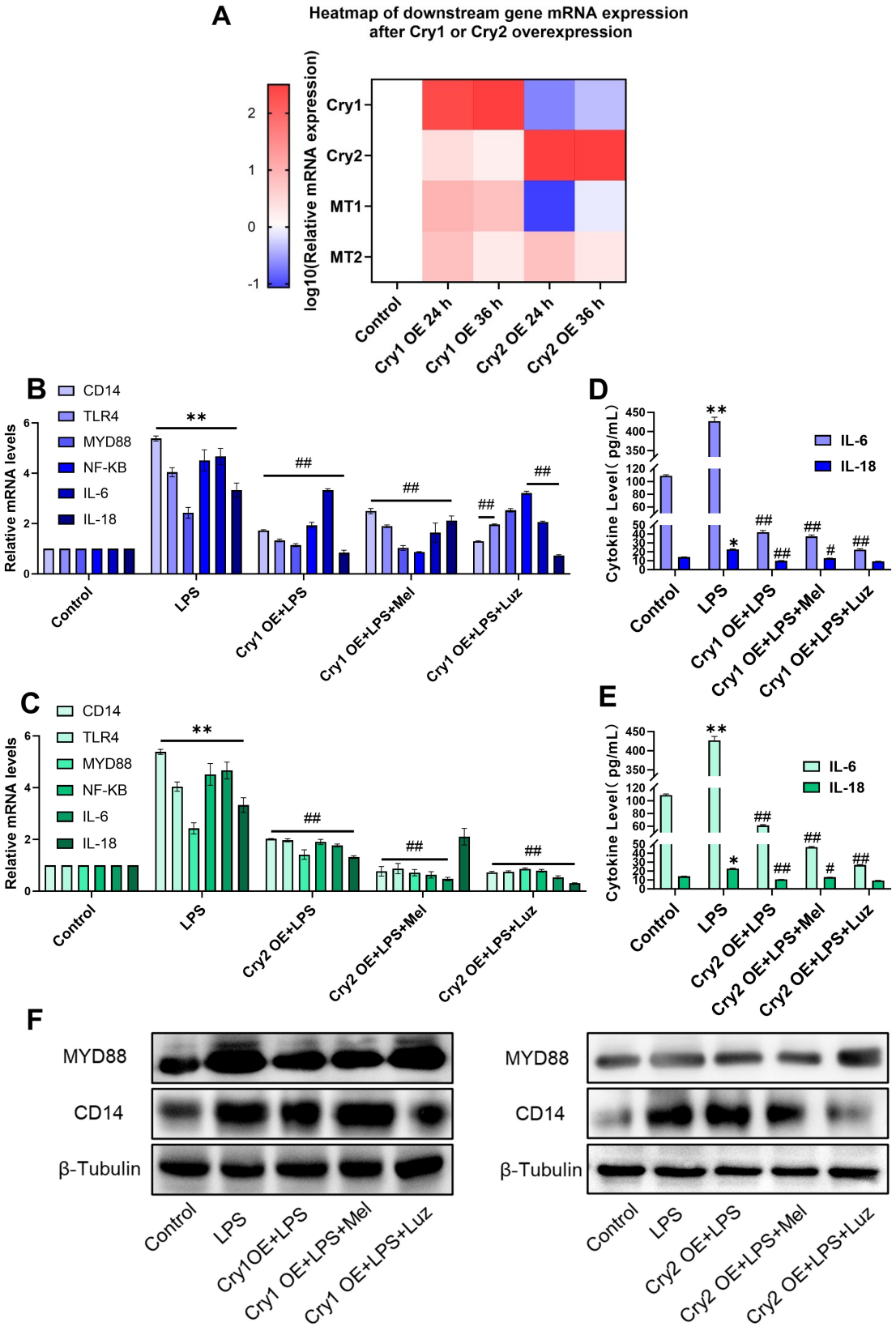

Fig. 3: Cry1/2 mediate anti-inflammatory effects by regulating MT1/2 signaling in Bactrian camel primary caput epididymis cells. (A): Differential regulation of *MT1* and *MT2* expression by *Cry1/2*. (B): The expression of inflammation-related genes after overexpression *Cry1*. (C): The expression of inflammation-related genes after overexpression *Cry2*. (D): ELISA measurement of IL-6 and IL-18 secretion after overexpression *Cry1*. (E): ELISA measurement of IL-6 and IL-18 secretion after overexpression *Cry2*. (F): Western blot analysis of CD14 and MYD88 protein expression. Tubulin served as loading control. *Cry1* OE is for overexpression *Cry1*; *Cry2* OE is for overexpression *Cry2*; "Luz" is for the abbreviation of "Luzindole"; "Mel" is for the abbreviation of "Melatonin". Compared with control group: *P<0.05, **P<0.01; compared with LPS group: #P<0.05, ##P<0.01.

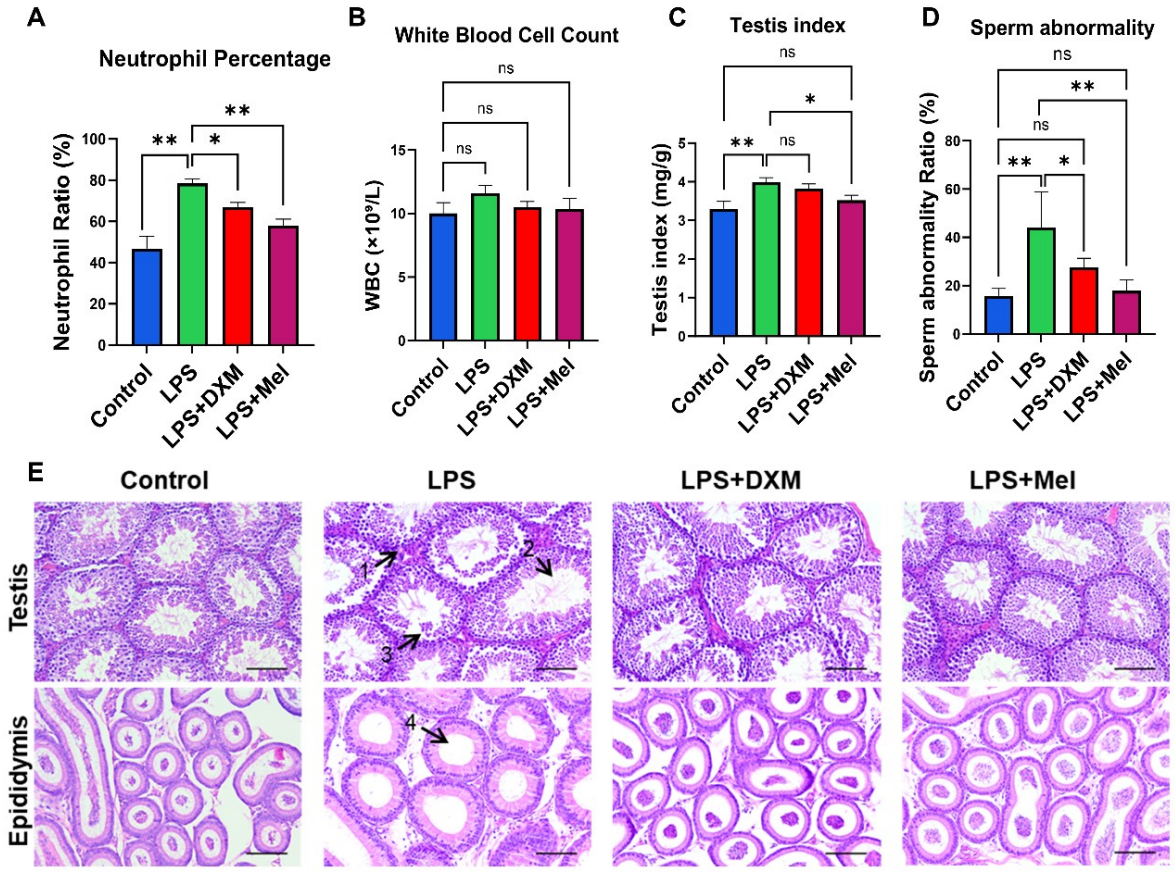

Fig. 4: Melatonin ameliorates LPS-induced peripheral blood inflammation, testicular injury, and sperm abnormalities in mice. (A): Percentage of neutrophils (NEU%). (B): Total white blood cell count (WBC, ×10^9/L). (C): Testicular index (unilateral testis weight/body weight, mg/g). (D): Sperm abnormality rate. (E): Histopathological alterations in testicular and epididymal tissue (H&E staining, 200×). Numbers indicate pathological changes: (1) disruption of seminiferous tubules; (2) decreased sperm count; (3) shedding and exfoliation of germ cells; and (4) reduction in sperm and secretory components. Scale bar = 100 μm. "Mel" is the abbreviation of "Melatonin"; "DXM" is the abbreviation of "dexamethasone". Statistical analysis: *P<0.05, **P<0.01; ns, non-significant.

level of these genes in the epididymis than in the LPS group (P<0.01). In the epididymis, the expression levels of *Nectin3* and *PRM1* in the LPS + Mel group were higher than those in the LPS+DXM group. Thus, melatonin showed a stronger promoting effect on *Nectin3* and *PRM1* expression than DXM (Fig. 5F).

Effect of *Cry1* overexpression combined with melatonin on epididymo-orchitis: In the present study, in vitro cell experiments demonstrated that melatonin enhanced the anti-inflammatory effects after *Cry1/2* overexpression. When pEGFP-*Cry1* recombinant plasmid was subcutaneously injected into the scrotum of mice and melatonin was administered by gavage, it was observed that overexpression of *Cry1* increased *MT2* in the testis (Fig. 6A), and melatonin primarily compensated it by enhancing *MT1/2*. In the epididymis (Fig. 6B), *Cry1* overexpression strongly upregulated *MT1*, while LPS abnormally upregulated both *MT1* and *MT2*. Combined melatonin treatment restored the *MT1/2* balance. These results highlight tissue-specific cooperative regulation by melatonin and *Cry1*.

Furthermore, LPS markedly increased the mRNA expression of inflammatory mediators in testes (Fig. 6C) and epididymis (Fig. 6D). LPS significantly increased the expression of *CD14*, *TNF-α*, *IL-6* and *IL-1β* compared to control (P<0.01), increased MYD88 expression was primarily observed in the epididymis (Fig. 6D). Compared with the LPS group, *Cry1* overexpression significantly suppressed most of these inflammatory genes in both tissues (P<0.05), with the exception of *CD14* and *IL-6* in the testis. Consistent with the RT-qPCR results, protein expression of CD14 and MYD88 was increased following LPS exposure, while Cry1 overexpression combined with melatonin treatment effectively reduced their levels (Fig. 6E). In addition, LPS significantly downregulated (P<0.01) the expression of germ cell structure and maturation-related genes (*Nectin3*, *PRM1*, and *TH2B*) compared to controls in the epididymis (Fig. 6F), whereas non-significant changes were observed *Nectin3* and *PRM1* expression in the testis (Fig. 6G). Compared with LPS alone, combined *Cry1* overexpression and melatonin treatment more effectively restored epididymal gene expression than melatonin treatment alone, especially in restoring epididymal function.

DISCUSSION

The bidirectional and environment-dependent immunomodulatory effects of melatonin: Findings of the current study revealed that in the absence of inflammatory stimulation, the effect of melatonin exhibited

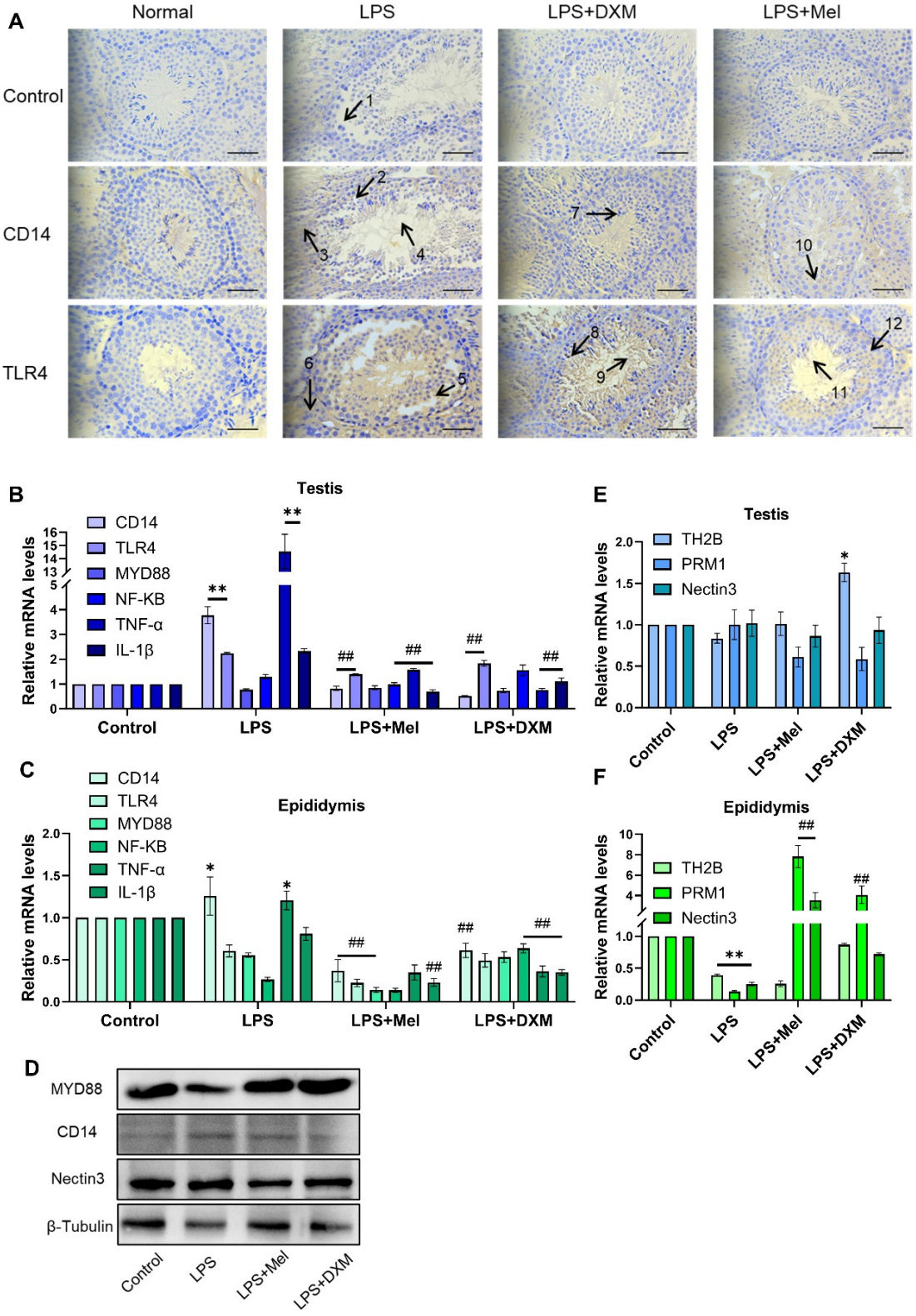

Fig. 5: Melatonin alleviates LPS-induced inflammatory responses and spermatogenic impairment by regulating the TLR4/NF-κB signaling pathway and spermatogenesis-related genes. (A): Immunohistochemical staining of CD14 and TLR4; Numbers indicate: (1, 2, 5) vacuolated intraluminal cell disruption; (3, 6) CD14 and TLR4 expression; (4) reduced sperm count; (7, 8) mild intraluminal cell rupture; (9, 11) sperm quantity; (10, 12) disappearance of vacuolization. Scale bar = 50μm. (B): Relative mRNA expression levels of inflammation-related genes in mice testes. (C): Relative mRNA expression levels of inflammation-related genes in mice epididymides. (D): Western blot analysis of CD14, MYD88, and Nectin3 protein expression in testes. Tubulin served as loading control. (E): Relative mRNA expression levels of spermatogenesis-related genes in mice testes. (F): Relative mRNA expression levels of spermatogenesis-related genes in mice epididymides. "Mel" is for the abbreviation of "Melatonin"; "DXM" is for the abbreviation of "dexamethasone". Compared with control group: *P < 0.05, **P<0.01; compared with LPS group: #P<0.05, ##P<0.01.

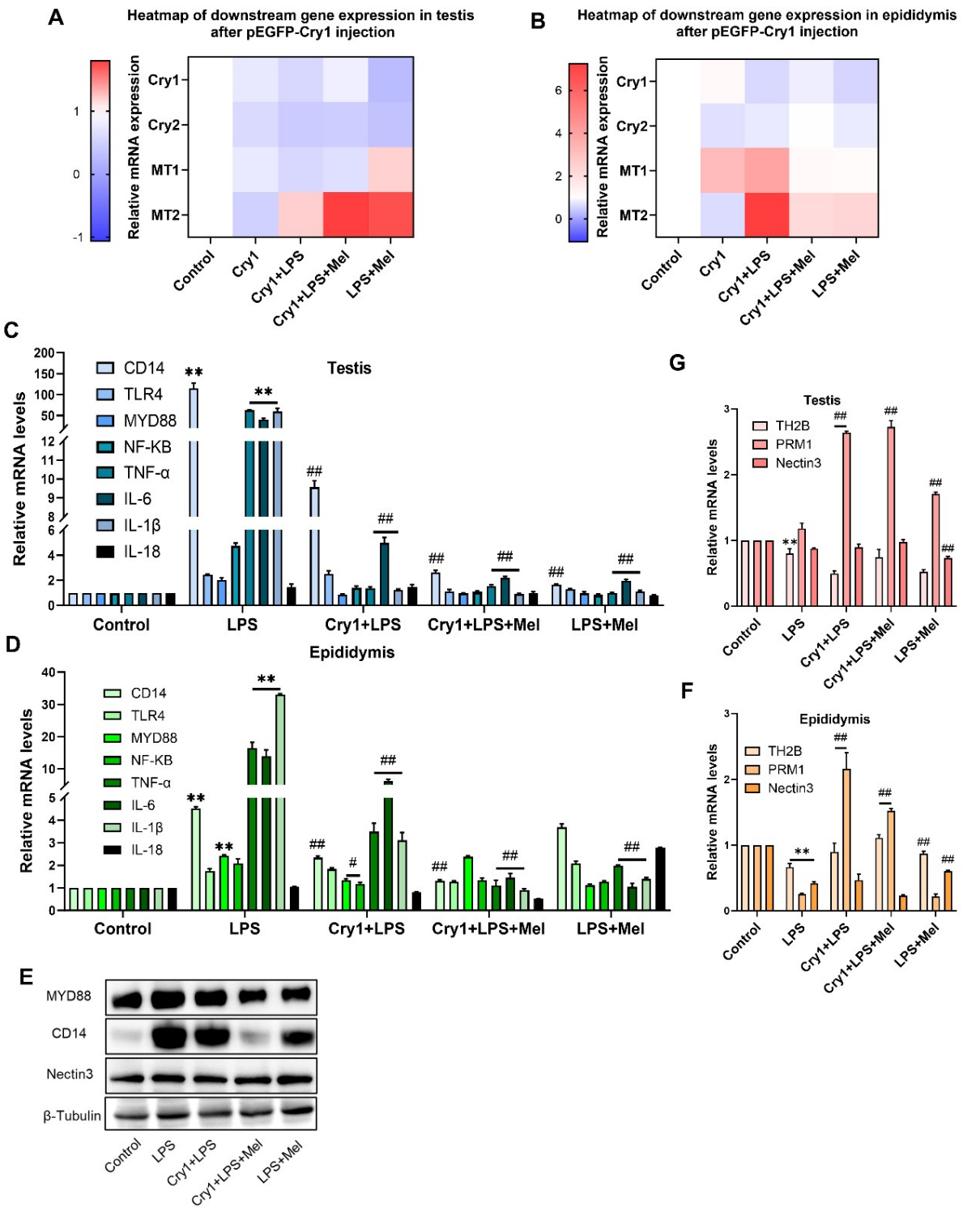

Fig. 6: Combined effects of *Cry1* and melatonin in the mice epididymo-orchitis model. (A): The *Cry1*, *Cry2*, *MT1*, and *MT2* mRNA expression of testes after pEGFP-*Cry1* injection into mice scrotum. (B): The *Cry1*, *Cry2*, *MT1*, and *MT2* mRNA expression of epididymides after pEGFP-*Cry1* injection into mice scrotum. (C): Relative mRNA expression levels of inflammation-related genes (*CD14, TLR4, MYD88, NF-κB, TNF-α, IL-6, IL-1β, IL-18*) in testes. (D): Relative mRNA expression levels of inflammation-related genes (*CD14, TLR4, MYD88, NF-κB, TNF-α, IL-6, IL-1β, IL-18*) in epididymides. (E): Western blot analysis of CD14, MYD88, and Nectin3 protein expression in testes. Tubulin served as loading control. (F): Relative mRNA expression levels of *Nectin3*, *PRM1*, and *TH2B* in epididymides. (G): Relative mRNA expression levels of *Nectin3*, *PRM1*, and *TH2B* in testes. *Cry1* OE is for overexpression *Cry1*; "Mel" is the abbreviation of "Melatonin". Compared with control group: *P<0.05, **P<0.01; compared with LPS group: #P<0.05, ##P<0.01.

two opposite patterns over time. It first promoted an increase in the expression level of inflammation-related genes and then inhibited them. This indicates that melatonin may play the role of an "immune buffer factor", enhancing the vigilance of innate immunity in homeostasis and inhibiting overresponse during inflammation (Hardeland, 2018).

Previous studies have also pointed out that melatonin can continuously exert anti-inflammatory effects even in a steady-state environment, and no early pro-inflammatory stage has been observed. For instance, clinical trials have shown that melatonin supplementation reduces the levels of pro-inflammatory cytokines such as IL-1 and IL-6, demonstrating its sustained anti-inflammatory effect (Cho *et al*., 2021). Differences in cell type, dosage, treatment duration, or receptor expression may explain the discrepancies between our findings and those of Radogna *et al*. (2010), where melatonin was described as a pleiotropic immunomodulator capable of exhibiting both anti-inflammatory and pro-inflammatory effects, depending on the stage of inflammation and the cellular environment. Moreover, concentration-dependent effects of melatonin have been recorded. At relatively low or physiological concentrations, melatonin may briefly activate certain immune responses, which also helps in the control of inflammation. In contrast, higher or pharmacological concentrations of melatonin primarily inhibited excessive inflammatory signaling by reducing oxidative stress and inhibiting NF-κB-dependent pro-inflammatory mediators (Luchetti *et al*., 2010). Thus, our results underscore a unique, dose- and time-sensitive immunoregulatory role for melatonin.

Role of MT receptors and Cry proteins in melatonin-mediated anti-inflammatory effects: The anti-inflammatory effects of melatonin are critically dependent on MT1/2 receptors. Our results showed that when the receptor antagonist luzindole was administered, the inhibitory effect of melatonin on inflammatory mediators was weakened, almost reaching the levels of the LPS-only group. This is consistent with previous studies demonstrating that blocking melatonin signaling exacerbates inflammation (Ali *et al*., 2020; Matos *et al*., 2021; Yan *et al*., 2024). Conversely, according to some other studies, melatonin may function independently of receptors, for example, by scavenging free radicals or regulating nuclear receptors (Xu *et al*., 2018). Therefore, the regulation of inflammation by melatonin is very likely to involve both receptor-dependent and receptor-independent mechanisms simultaneously.

The present research further revealed the role of *Cry1* and *Cry2* in melatonin signaling. Increasing the expression of *Cry1* can significantly inhibit the expression of inflammatory genes caused by LPS, especially through the TLR4/NF-κB pathway. These results are supported by those of some earlier studies suggesting that cryptochrome proteins are involved in controlling inflammatory responses (Hashiramoto *et al*., 2010; Narasimamurthy *et al*., 2012; Yang *et al*., 2015).

In addition, melatonin can enhance the inhibitory effect of *Cry1* in a synergistic manner, while the use of luzindole can weaken these effects. This indicates that the action of *Cry1* partly depends on the MT receptor pathway, which is also consistent with our earlier observation that *Cry1* can up-regulate *MT1/MT2* transcription (Zhao *et al*., 2025). In contrast, the performance of *Cry2* was different: although melatonin enhanced its anti-inflammatory effect, when melatonin was used in combination with *Cry2*, the expression of certain cytokines was abnormally increased, and luzindole further strengthened the inhibition caused by *Cry2*. These results suggest that *Cry2* may be negatively regulated by MT receptor signaling.

Melatonin has demonstrated comprehensive protective effects in animal models: In the mice epididymo-orchitis model, melatonin not only exhibited an anti-inflammatory effect comparable to that of dexamethasone, but also maintained the structural integrity of reproductive tissues. Specifically, melatonin restored the expression level of genes influencing sperm production and reduce sperm abnormalities. These results indicate that melatonin can not only inhibit inflammation but also alleviate reproductive function damage caused by inflammation, which is supported by the results of previous studies (Li *et al*., 2016; Zi *et al*., 2022; Zhao *et al*., 2023). Although traditional glucocorticoids have strong anti-inflammatory effects, their long-term application can show adverse effects. In contrast, melatonin is a safer option, as it has both anti-inflammatory and reproduction protective effects.

In this study, the interaction between circadian clock protein cryptochrome and melatonin receptors MT1 and MT2 was primarily inferred by changes in gene and protein expression levels, while direct molecular interactions have not been studied, and the specifics of this remain to be elucidated. Additionally, melatonin's effects were evaluated at selected doses, while exploration over a broader range of concentrations may provide a more comprehensive understanding of its immunomodulatory dynamics. Future research on these aspects will help to further elucidate the mechanism of action of the Cry–MT1/2 module in inflammation-related reproductive diseases.

Conclusions: Epididymo-orchitis in Bactrian camels threatens reproductive function. In the present study, short-term (6 h) melatonin treatment appeared to be pro-inflammatory, while prolonged (24 h) treatment showed anti-inflammatory effects via MT1/2-dependent TLR4/NF-κB signaling pathway inhibition. Combined *Cry* and melatonin treatment was more effective than melatonin alone. In a murine model, melatonin reduced peripheral inflammation, improved testis and epididymis pathology, and restored sperm quality. Compared with dexamethasone, melatonin showed better effects on germ cell development and cell adhesion genes (*Nectin3*, *PRM1*, *TH2B*). Furthermore, *Cry1* plus melatonin showed synergistic benefits. These results reveal the role of melatonin in linking inflammation and clock genes, suggesting new targets for epididymo-orchitis therapy.

Ethical approval: All animal procedures followed the guidelines of the Animal Welfare and Ethics Committee of Gansu Agricultural University (GAU-LC-2022-33), China.

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Authors contribution: SZ conceived the idea, supervised the study, and made necessary revision of the manuscript. SJ performed the experiments and prepared the manuscript. ML, JH, YF, CW and YG contributed to data analysis. SZ and SJ contributed equally to this work. All authors discussed the results, reviewed the manuscript, and approved the final version for submission.

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