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Melatonin alleviates circadian rhythm disruption exacerbating DSS-induced colitis by inhibiting the distribution of HMGB1 in intestinal tissues

Xi-Wen Liu, Cheng-Dang Wang*

The First Affiliated Hospital of Fujian Medical University, 20 Chazhong Road, Fuzhou, Fujian, PR China

A B S T R A C T

Circadian rhythm disruption (CRD) is regarded as a risk factor for inflammatory bowel disease (IBD), and it was reported to suppress the level of melatonin, which execute anti-inflammatory effects. High mobility group box 1 protein (HMGB1) is a member of the damage-associated molecular pattern (DAMP) family and has been verified as an IBD-associated inflammatory cytokine that mediates the TLR4-NF-κB pathway. However, no exact mechanism has been illustrated among melatonin, disrupted circadian rhythm and inflammatory bowel disease, as well as regarding the effect of melatonin on HMGB1. In the present study, we aimed to explore the role of relationship with HMGB1. CRD aggravated DSS-induced colitis by worsening colonic inflammation and tissue injury, as well as by enhancing HMGB1 translocation, which could be reversed by ethyl pyruvate, an HMGB1 antagonist. Moreover, melatonin treatment attenuated these disorders and the shuttling of HMGB1 in the intestinal epithelial cells (IECs), the effect of which could be partly reversed by the melatonin antagonist luzindole. The protective role of melatonin may be tightly related to the translocation of HMGB1 in IECs. Accordingly, these results suggested that melatonin may be a new therapeutic beneficial option in IBD patients, especially for those with circadian rhythm disruption.

1. Introduction

Inflammatory bowel disease (IBD) is characterized by recurrent diarrhoea, abdominal discomfort, rectal bleeding, and weight loss, of which Crohn's disease (CD) and ulcerative colitis (UC) are two principal types. Various factors contribute to the development of IBD, including environmental factors, genetics, host and microbiota [1]. Although it was observed that the incidence of IBD is higher in western countries, a rapid increase has also been seen in low-incidence Asian areas.

Meanwhile, data based on the IBD population showed that a family history of IBD is less common in Asia [2]. Additionally, relevant studies in genetic analysis revealed that only 25% of the already known alleles or genes have been identified with a heritable risk of IBD that could hardly change in a few decades [3]. Therefore, high value has been placed on environmental contributions [4].

Light is an irreplaceable environmental influence for circadian rhythm and sleep via the light-dark cycle as well as the sleep-wake cycle. Extra light exposure or irregular day and night lifestyles disturb sleep and circadian rhythm, which may lead to the imbalance and aggravation of inflammation. A large retrospective study of > 12,000 people suggested that the incidence of IBD is higher in people with irregular working hours than in regular workers [5].

Additionally, a study based on the Nurses Health Survey indicated that women with sleep duration of fewer than 6 h/day (11/100,000 person-years) or > 9 h/day (20/100,000 person-years) had a higher ulcerative colitis incidence rate ($P < 0.05$) [6]. Animal experiments showed that circadian rhythm disruption (CRD) or sleep disruption contributed to the deterioration of IBD. Tang et al. [7] found that C57BL/6 mice under abnormal light conditions appeared to show increased sensitivity to dextran sulphate sodium salt (DSS), resulting in worse disease activity analysis, higher histological scores and oxidative stress. Summa et al. [8] further investigated that phase shifts increased the permeability of the colonic intestinal mucosal barrier in DSS mice.

Melatonin is an important endocrine hormone released by the brain's pineal gland; however, recently, its secretion by peripheral organs such as the enterochromaffin cells of the GI mucosa was also found [9]. Melatonin exerts the function of synchronizing biological rhythm and promotes night time sleep. Evidently, melatonin production could be suppressed not only by bright light but also by monochromatic light at wavelengths of 440 to 460 as low as 1 lx or less, as can < 100 lx of broad-spectrum fluorescent light [10–12]. Additionally, these light levels help to induce significant phase shifts of the circadian clock and sleep disturbance that are commonly seen in CRD model buildings [7,8,13,14]. Furthermore, melatonin is beneficial to enhance immunity as well as the antioxidant potential. Previous reports have noted that melatonin exerts an anti-inflammatory effect in animal models of colitis [15–17]. However, no exact mechanism has been illustrated among melatonin, disrupted circadian rhythm, and its relation to inflammatory bowel disease.

High mobility group box 1 protein (HMGB1) is one of the most

* Corresponding author at: The First Affiliated Hospital of Fujian Medical University, Fuzhou, Fujian Province 350005, China.
E-mail address: 1786872326@qq.com (C.-D. Wang).

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important chromatin proteins that organizes DNA and regulates transcription. This member of the damage-associated molecular pattern (DAMP) family can be secreted as a cytokine mediator of inflammation by activated macrophages and monocytes, followed by binding to Toll-like receptors (TLRs). The combination of HMGB1 and TLR4 initiates signal transduction and the activation of various signalling cascades, including the NF-κB pathway, resulting in the production of inflammatory cytokines [18,19]. Many studies demonstrated that HMGB1-mediated inflammation would be inhibited by melatonin in certain animal models, such as brain damage [20], sepsis-induced cardiac dysfunction [21] and liver fibrosis [22]. However, few investigations are related to disrupted circadian rhythm, as well as to impaired melatonin, and HMGB1 in inflammatory bowel disease. Additionally, some studies have reported that the protein HMGB1 has diurnal rhythm [23], which may indicate the relationship between circadian rhythm disorder and HMGB1-mediated inflammation.

Therefore, we hypothesized that a disrupted circadian rhythm will deteriorate colitis and decline the level of melatonin in DSS-induced animals, while melatonin treatment can reverse the disease by inhibiting the inflammation-induced function of HMGB1, and investigated whether there are diurnal changes in HMGB1 protein expression in intestinal tissue.

2. Methods and materials

2.1. Experimental design

The experimental procedures were reviewed and approved by the Institutional Animal Care Committee of the First Affiliated Hospital of Fujian Medical University, China. All experiments were performed on eight-week-old C57BL/6J male mice (22–25 g). The mice were purchased from Vital River Laboratory Animal Technology (Beijing, China) and were housed individually under a regular 12:12-h light-dark (rhythmic) cycle at approximately 22 °C with free access to food and water for 2 weeks.

They were randomized into 7 groups (6 mice per group): group 1, circadian rhythm disruption (CRD); group 2, 2% dextrose sodium sulphate (DSS; MW:36000-50000; MP Biomedicals Inc., CA, USA)-induced colitis with CRD; group 3, DSS-induced colitis for 7 days; group 4, control; group 5, colitis with CRD, melatonin and luzindole; group 6, colitis with CRD and ethyl pyruvate; group 7, colitis with CRD and melatonin. They were then divided into 2 parts, the first part including groups 1–4 and the second part including group 2 and groups 5–7.

2.2. Circadian rhythm desynchronization

For circadian rhythm disruption (CRD), the mice were singly randomized into the phase-shift groups under a condition of once weekly 12-h phase shifts of the LD cycle for 2 months. Animals kept under a constant 12:12 LD cycle condition remained as controls.

Locomotor activity levels were measured by monitoring infrared beam break. Briefly, an infrared beam is broken, and the activity is recorded using running wheels installed in each cage. The mice were regarded as circadian rhythm disrupted when statistical analysis of the locomotor activity revealed circadian rhythm disorder for > 5 consecutive days. Food intake and body weight were recorded for the duration of the experiment.

2.3. Induction of colitis and pharmacological treatments

The mice were administered 2% dextran sodium sulphate (DSS; MW36000-50000; MP Biomedicals) in the daily drinking water for 7 days [24]. The remaining animals in each group received tap water without DSS as a control.

After CRD was observed in mice with 2% DSS-induced colitis, the mice of groups 5, 6, and 7 received pharmacological treatments as mentioned above.

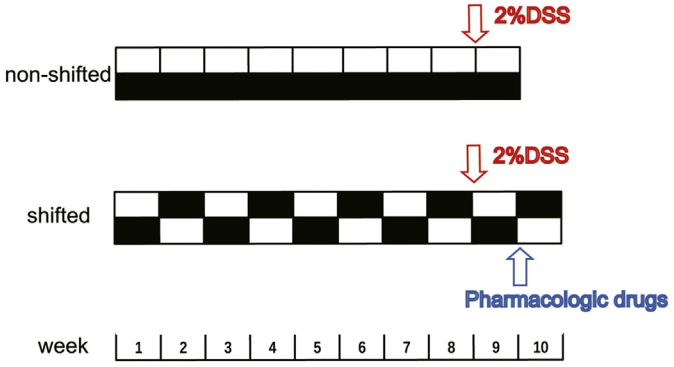

Fig. 1. Experimental protocols of circadian disruption, colitis induction and pharmacological treatments. C57BL/6J male mice (22–25 g) were individually housed under a regular 12:12-h light-dark (LD, rhythmic) cycle for 2 weeks. The mice were randomly assigned to either a phase-shift group under the condition of a once weekly 12-h phase shift of the LD cycle or a constant 12:12 LD cycle condition for 2 months. The black block indicates night, while the white block indicates day.
In part 1, shifted or non-shifted mice were either administered 2% dextran sodium sulphate (DSS) in the daily drinking water for 7 days or tap water, resulting in four experimental groups: control (CT), DSS, circadian rhythm disruption (CRD) and CRD with DSS groups. At the end of week 8, the mice were euthanized.
In part 2, CRD with DSS mice were randomized into three pharmacological schedules after 8 weeks, resulting in three experimental groups: melatonin treatment, melatonin and luzindole treatment, and ethyl pyruvate treatment. At the fourth day of week 10, the mice were euthanized.

Melatonin (Sigma, US) was dissolved in ethanol, diluted in phosphate-buffered saline (the final concentration of ethanol was 1%) and then was administered to the mice at a dose of 10 mg/kg for 4 days intraperitoneally.

Luzindole (Sigma, US) was dissolved in 5% dimethyl sulfoxide (DMSO) in saline and then administered i.p. at a dose of 5 mg/kg 15 min prior to melatonin injection.

Ethyl pyruvate (Sigma, US) in lactated Ringer's solution in a total volume of 500 μl was administered i.p. at a dose of 40 mg/kg for 4 days intraperitoneally.

The detailed experimental procedure is depicted in Fig. 1A.

Locomotor activity and water intake, as well as body weight, were recorded throughout the whole experiment.

2.4. Sample collection and analysis

For animals kept on a 12:12 LD cycle, the Zeitgeber time (ZT) 0 shows the time of the start of light, while ZT 12 indicates the time of lights off. Animals were euthanized between ZT 4 and 6. Colon tissue was harvested, and the colon length was measured from the end of the caecum to the anus. Blood was collected for serum detection.

2.5. Histology and immunohistochemical analysis

Colon tissues were fixed in 4% formaldehyde solution. The fixed tissues were first dehydrated, embedded and then sliced into 4-μm sections that were stained with haematoxylin and eosin to evaluate the histological changes. Five fields per section were examined to determine the morphological lesions and changes in the colon mucosa. Histological scoring for intestinal inflammation of the colon was performed by gastroenterological pathologists in a blinded way using a validated scoring system [7] (Table 1).

Tissue sections were subjected to immunohistochemical analysis with heat antigen retrieval. Endogenous peroxidase was blocked with EDTA (pH 8) for 20 min, and nonspecific antigens were blocked with

Table 1
Histological criteria for the degree of gastrointestinal inflammation.

Criteria	0	1 (%)	2 (%)	3 (%)	4 (%)
Goblet cell loss	0	0–25	25–50	50–75	75–100
Mucosal thickening	0	0–25	25–50	50–75	75–100
Inflammatory cell number	0	0–25	25–50	50–75	75–100
Submucosal cell infiltration areas	0	0–25	25–50	50–75	75–100
Disruption of architecture	0	0–25	25–50	50–75	75–100
Ulcers	0	0–25	25–50	50–75	75–100

Note: The criteria for intestinal inflammation are modifications of the criteria used by Tang et al. [7].

serum for 30 min. The slides were stained with rabbit anti-HMGB1 (1:100; Servicebio Technology Co., Wuhan, China), rabbit anti-TLR4 (1:100; Abcam, UK) and F-actin (1,50; Novus Biologicals, USA) antibodies at 4 °C overnight. The samples were then incubated with fluorescent-labelled secondary antibodies for 1 h at room temperature.

2.6. Western blot analysis

Briefly, total proteins were obtained from colon tissue, and the concentrations were quantified using a bicinchoninic acid protein concentration determination kit (Beyotime Institute of Biotechnology, Beijing, China). The proteins were separated using 10% SDS-PAGE and then were transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk in TBST at room temperature and were incubated with antibodies against HMGB1 (1:1000; Servicebio, China), MyD88 (1:200; Abcam, UK), TLR4 (1:500; Abcam, UK), NF-κB (1:1000; Servicebio, China) and β-actin (1:1000; Servicebio, China) overnight at 4 °C. Followed by 3 washes with TBST for 10 min, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:2000; OriGene Technologies, Inc., Beijing, China) for 1 h at room temperature. After another 3 washes with TBST for 10 min each time, the protein bands were visualized using a Bio-Rad imaging system (Bio-Rad, Hercules, CA, USA) and were quantified using Image Pro Plus version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

2.7. Evaluation of faecal HMGB1 and inflammatory cytokines

The faecal samples of sacrificed mice were collected in PBS by rotation at 4 °C for 48 h and were analysed for the HMGB1 concentration by ELISA. The HMGB1 ELISA kit was purchased from R&D Systems.

The inflammatory cytokines in the serum and cortex were measured 48 h after surgery by commercially available TNF-α, IL-1β, and MPO ELISA kits. The data were analysed using a microplate reader (Multiskan Spectrum; Thermo Scientific).

2.8. Statistical analysis

Statistical analysis was conducted using Prism 7.0 (GraphPad, La Jolla, CA, USA). The data were presented as means ± standard error (SD). Values of $P < 0.05$ were considered to be significantly different. Collective comparisons were carried out by ANOVA (one-or two-way analysis of variance) with Levene's test and Tukey's multiple comparison tests.

3. Results

3.1. Impaired circadian rhythm in phase-shift mice

Phase-shifted animals showed CRD compared with that in the control group (Fig. 2A). There was no significant difference in the body weight, water intake, colon length and inflammatory markers, as well as the inflammatory cytokines, histopathology scores and protein levels, between CRD mice and the matched group (Fig. 2B–F, Fig. 3), indicating that CRD alone neither causes obvious weight loss nor induces colitis.

3.2. DSS induces systemic and colonic inflammation

DSS (2%) given in the drinking water for 7 days led to severe colitis (approximately 22.6% weight loss compared with that in the control group) but no death in mice. Animals lost weight from the fourth days of receiving DSS water and deteriorated until the end of the whole process (Fig. 2B).

Additionally, the colon length showed obvious shortening (Fig. 2C), indicating the development of colitis. Histologic analysis revealed moderate inflammatory infiltrate and structural damage (Fig. 2D, E).

Elevated levels of TNF-α (increase by 1 time) and IL-1β (increase by 6 times) were found (Fig. 3C–D) that are important markers of systemic inflammation. Specifically, enhanced faecal HMGB1 (increase by 5 times) is an indicator of colitis (Fig. 3A). Furthermore, a marked increase in the protein expression of HMGB1 (90%), MyD88 (45%), TLR4 (62%) and NF-κB (89%) also illustrates colonic inflammation (Fig. 3E–H). Additionally, immunofluorescence demonstrated an increase in the nuclear and cytoplasmic distribution of HMGB1, as well as enhanced expression of TLR4 levels in the colonic epithelium of CRD, DSS, and CRD + DSS group, compared with that in the control group (Fig. 4).

3.3. CRD worsens colonic inflammation and tissue injury in DSS-induced colitis

CRD mice showed an aggravated course of colitis on DSS challenge, as reflected by deteriorative weight maintenance, as well as increased inflammatory markers [MPO was increased 3-fold, faecal HMGB1 was increased by 69%, and the protein levels of HMGB1 (44%), MyD88 (38%), TLR4 (33%), and NF-κB (48%) were upregulated compared with those in DSS-alone mice ($P < 0.05$)]. Additionally, compared with the control group, the histological scores increased in the colon of 2% DSS-induced colitis mice with CRD (Fig. 2C), resulting in higher levels of serum TNF-α (increase by 1-fold), IL-1β (increase by 9-fold), MPO (increase by 12-fold) as well as faecal HMGB1 (increase by 9-fold) (Fig. 3A–D) and up-regulated protein levels of HMGB1 (173%), MyD88 (100%), TLR4 (115%), and NF-κB (178%) (Fig. 3E–H).

3.4. Effect of pharmacological drugs on CRD mice with DSS-induced colitis

Melatonin (MEL) attenuated colitis symptoms in the CRD + DSS + MEL group compared with that in the CRD + DSS group ($P < 0.05$). Similarly, ethyl pyruvate plays the same role, although few differences were found regarding melatonin in the degree of alleviating colonic inflammation. However, luzindole abolished the protective effect of melatonin in the CRD + DSS + MEL + LUZ group (versus the group CRD + DSS + MEL, $P < 0.05$). Melatonin and ethyl pyruvate helped the mice to recover, and their weight was reversed to a certain extent (versus the CRD + DSS group; $P < 0.05$), while the luzindole group showed little effect (versus the CRD + DSS group; no significant difference) (Fig. 5B). Melatonin significantly improved the intestinal infiltration of inflammatory immune cells, mucosal injury such as oedema, ulcer or necrosis, which were indicated by the improved colon length and decreased histologic scores (versus the CRD + DSS group; $P < 0.05$). Ethyl pyruvate had a better effect than melatonin (versus the CRD + DSS group; $P < 0.05$) but luzindole reversed the effect (versus the CRD + DSS + MEL group; $P < 0.05$) (Fig. 5A, D).

Subsequently, the effects of pharmacological treatments on inflammatory cytokines in serum assessed that melatonin and ethyl pyruvate dramatically alleviated the levels of serum TNF-α (49% and 65%) and MPO (228% and 220%) respectively (versus the CRD + DSS group; $P < 0.05$). However, luzindole administration abolished the effect of

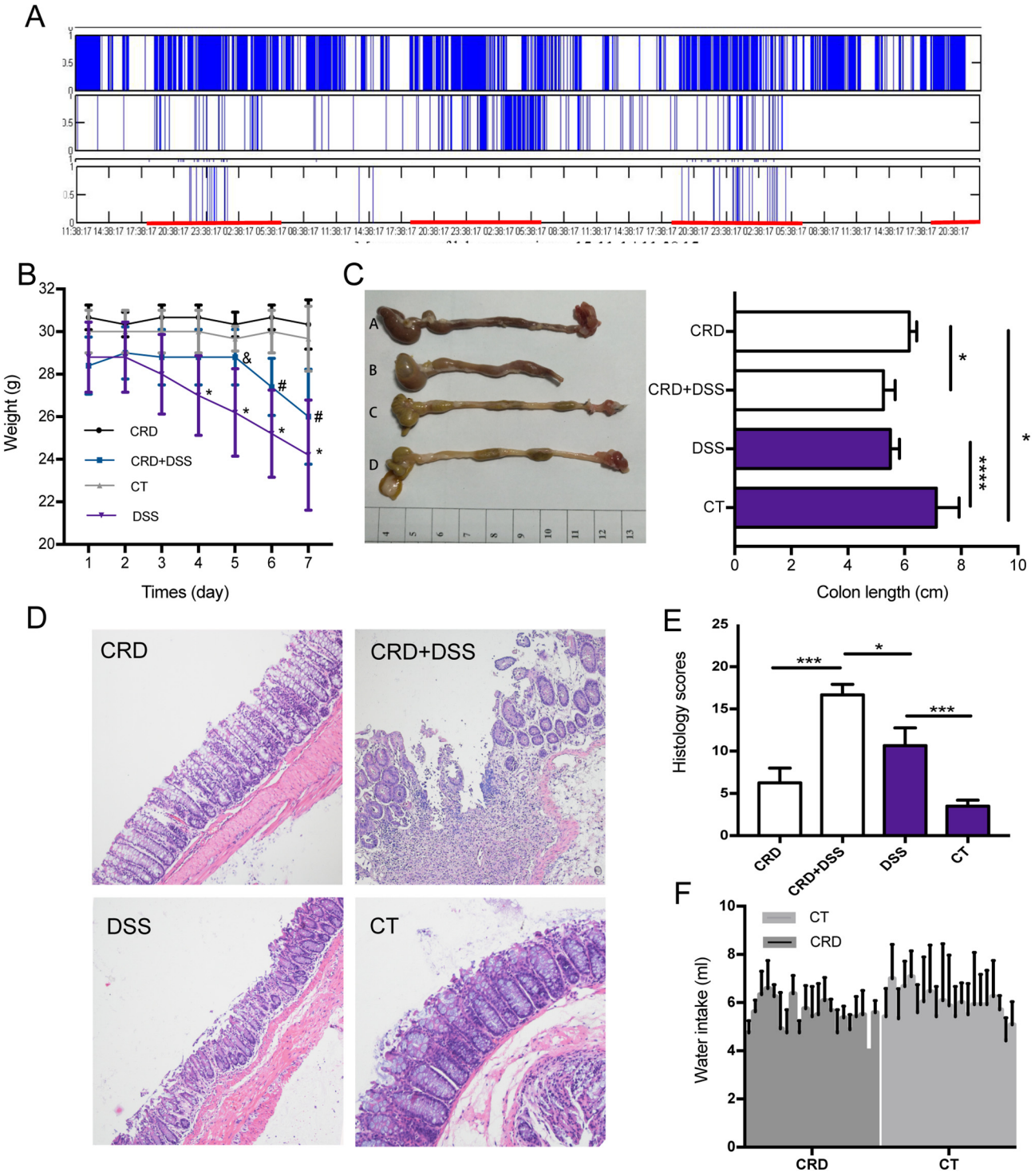

Fig. 2. Impaired circadian rhythm in phase-shifted mice colitis.
(A) The records of infrared beam breaks showing circadian rhythm disruption in upper line, in which the blue line shows animal activities and the red line indicates the night time. (B) Changes in the body weight. The results are expressed as the means ± SD ($n = 6$ in each group). *$P < 0.05$ compared with the control group, & $P < 0.05$ compared with the CRD + DSS group, #$P < 0.05$ shows significant a difference between the CRD + DSS group and CRD group. (C) Representative images of the colon length and (D) HE staining. Determination of the histological scores (E), as well as water intake (F). All values are means ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

melatonin in the CRD + DSS + MEL + LUZ group compared with the levels in the CRD + DSS + MEL group ($P < 0.05$) (Fig. 5C). Moreover, immunofluorescence demonstrated that melatonin and ethyl pyruvate dramatically alleviated the release of HMGB1 and TLR4, which were abolished by luzindole (Fig. 6).

Finally, the protein levels of HMGB1 (77% and 81%), MyD88 (537% and 274%), TLR4 (300% and 344%), and NF-κB (154% and 146%), as detected by Western blotting, indicated that melatonin and ethyl pyruvate moderately downregulated the expression of those proteins (versus the CRD + DSS group, $P < 0.05$). However, luzindole abolished the decrease in the expression of these proteins (versus the CRD + DSS + MEL group; $P < 0.05$) (Fig. 7).

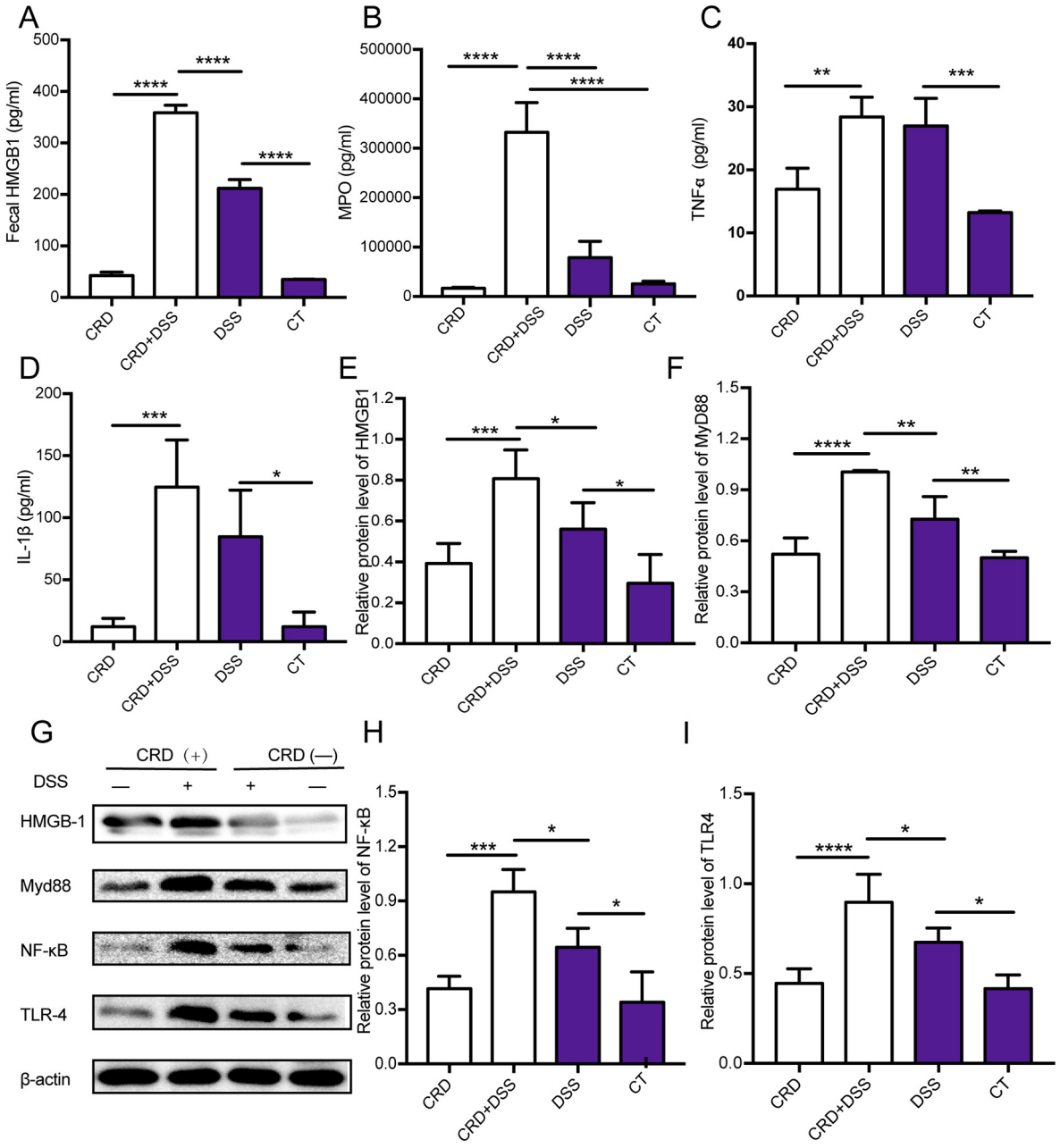

Fig. 3. CRD worsens colonic inflammation and tissue injury in DSS-induced colitis, as evaluated by ELISA and Western blot analysis. (A) Faecal HMGB1, (B) MPO activity, (C) TNF-α, and (D) IL-1 levels, as evaluated by ELISA. There are no significant difference between CRD + DSS vs DSS in Figures C and D. (G) Western blot analysis. These data were quantified, and relative amounts to the amount of β-actin are shown for the protein levels of E (HMGB1), F (MyD88), H(NF-κB)and I (TLR4). All values are means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

4. Discussion

The phase-shifted mice (circadian rhythm disruption mice, CRD mice) exhibited similar symptoms as the control. However, when the mice were presented with a DSS challenge to mimic intestinal inflammatory colitis, the CRD mice revealed more serious colitis symptoms such as body weight loss. The measurement of intestinal physiology and inflammatory indicators compared with non-phase shifted DSS-treated animals showed a significant difference.

Melatonin attenuated disorders in the CRD + DSS + Mel group compared with that in the CRD + DSS group, as indicated by the ameliorative body weight, colonic inflammation (levels of TNF-α, IL-1β, MPO, HMGB1, Myd88, TLR4, and NF-κB), and tissue injury. Similarly, the HMGB1 antagonist ethyl pyruvate showed few differences regarding the melatonin effects in the degree of alleviating colonic inflammation, whereas the melatonin antagonist luzindole abolished the protective effect of melatonin.

These data revealed that, in healthy control (non-DSS) mice, CRD had little influence on intestinal physiology, whereas phase shifting aggravated the severity of an existing disease state, as well as enhanced the colonic sensitivity to DSS challenge. Melatonin and the HMGB1 antagonist reduced the severity of UC in DSS-treated mice that was partly reversed by the melatonin antagonist luzindole.

Okayasu et al. [25] first proposed the DSS-induced colitis model, which was then extensively used to study colitis. In the present study, we utilized DSS-induced colitis and found that the time of weight loss in the CRD group was later than that of normal ones when they encountered a DSS challenge at the same time. After recording the water

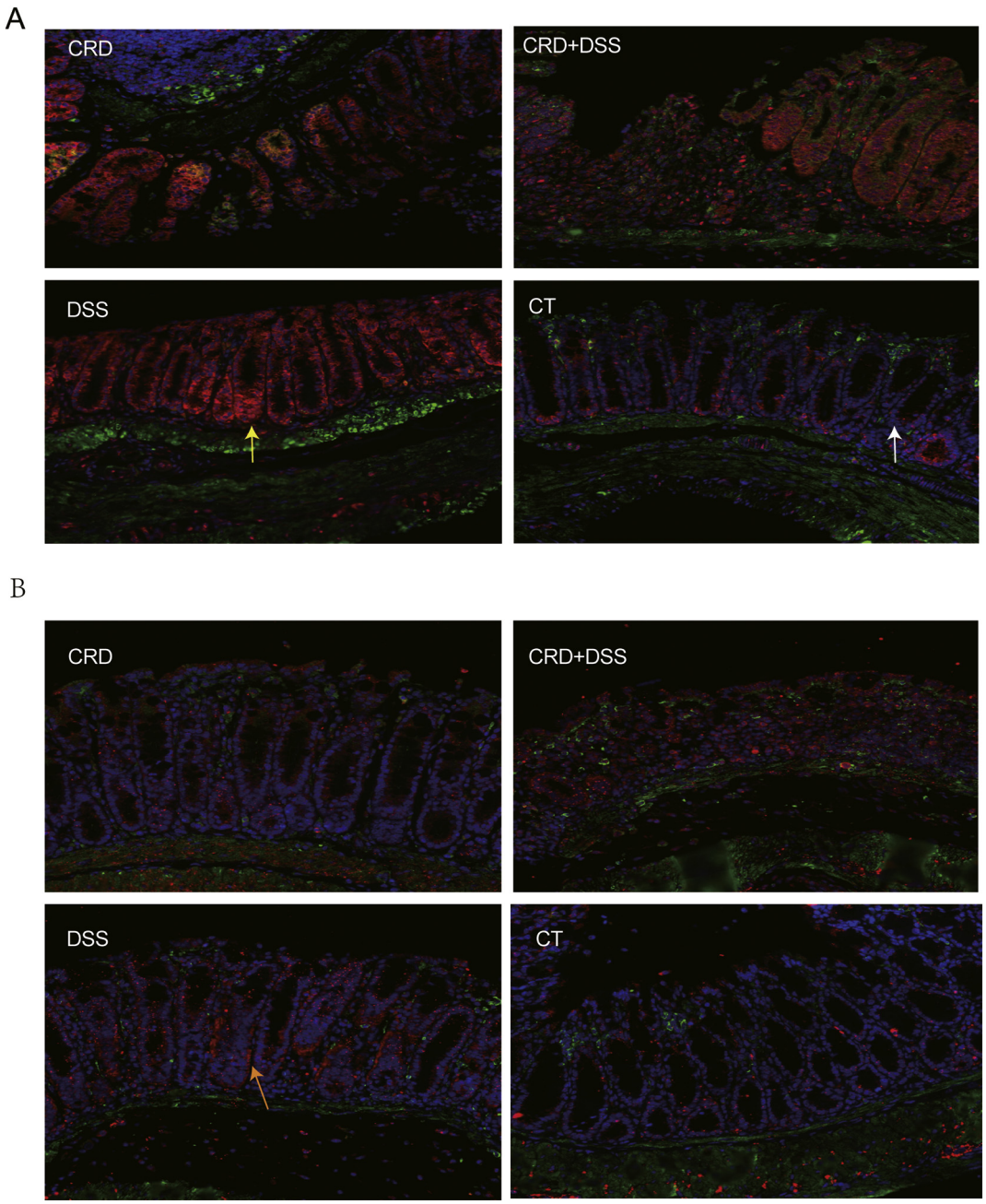

Fig. 4. CRD worsens colonic inflammation and tissue injury in DSS-induced colitis, as evaluated by immunochemical staining.
Paraformaldehyde-fixed colons were stained for actin (green), HMGB1 (red, yellow arrow in the picture), and nuclear Draq5 (blue, white arrow in the picture) in Fig. 4A while actin (green), TLR4 (red, orange arrow in the picture), and nuclear Draq5 (blue) were stained in Fig. 4B, followed by viewing under a fluorescence microscope. Immunofluorescence demonstrated an increase in the nuclear and cytoplasmic distribution of HMGB1, as well as enhanced expression of TLR4, in the colonic epithelium of the CRD, DSS and CRD + DSS groups compared with that in the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

intake of CRD and non-CRD mice for nearly a month, we discovered decreased water intake in the CRD group compared with that in the non-CRD group, but there was no significant difference between them. Similarly, a few studies have demonstrated analogous results while the mechanism remains unknown [26,27].

CRD has been reported to be related to the occurrence of metabolic disorders such as obesity, as well as diabetes, cardiovascular disease, and mood disorders [28–30]. Clinical studies also indicated a sleep disturbance in IBD patients, which is a type of CRD and an environmental predictor factor for the deterioration of IBD disease [31,32]. Additionally, some studies about CRD in IBD and the modelling methods included shifted L-D cycles and sleep deprivation. Preuss et al. [33] set up an animal model of shifted L-D cycles every 5 days for 3 months with DSS treatment to illustrate deleterious effects of CRD on UC mice. However, Tang et al. [7] demonstrated that intermittent sleep deprivation worsens the severity of colitis.

However, because no standard models of circadian rhythm exist, we implemented a complete reversal of the LD cycle every 7 days for 2 months to cause maximal disruption of the circadian organization and observed CRD by monitoring the mouse activities.

Melatonin is a hormone that was first discovered in 1958 and is involved in the entrainment course of the circadian rhythm [9]. Melatonin is produced in the pineal gland and is also generated by enterochromaffin cells (ECs) in the gastrointestinal (GI) tract, where its

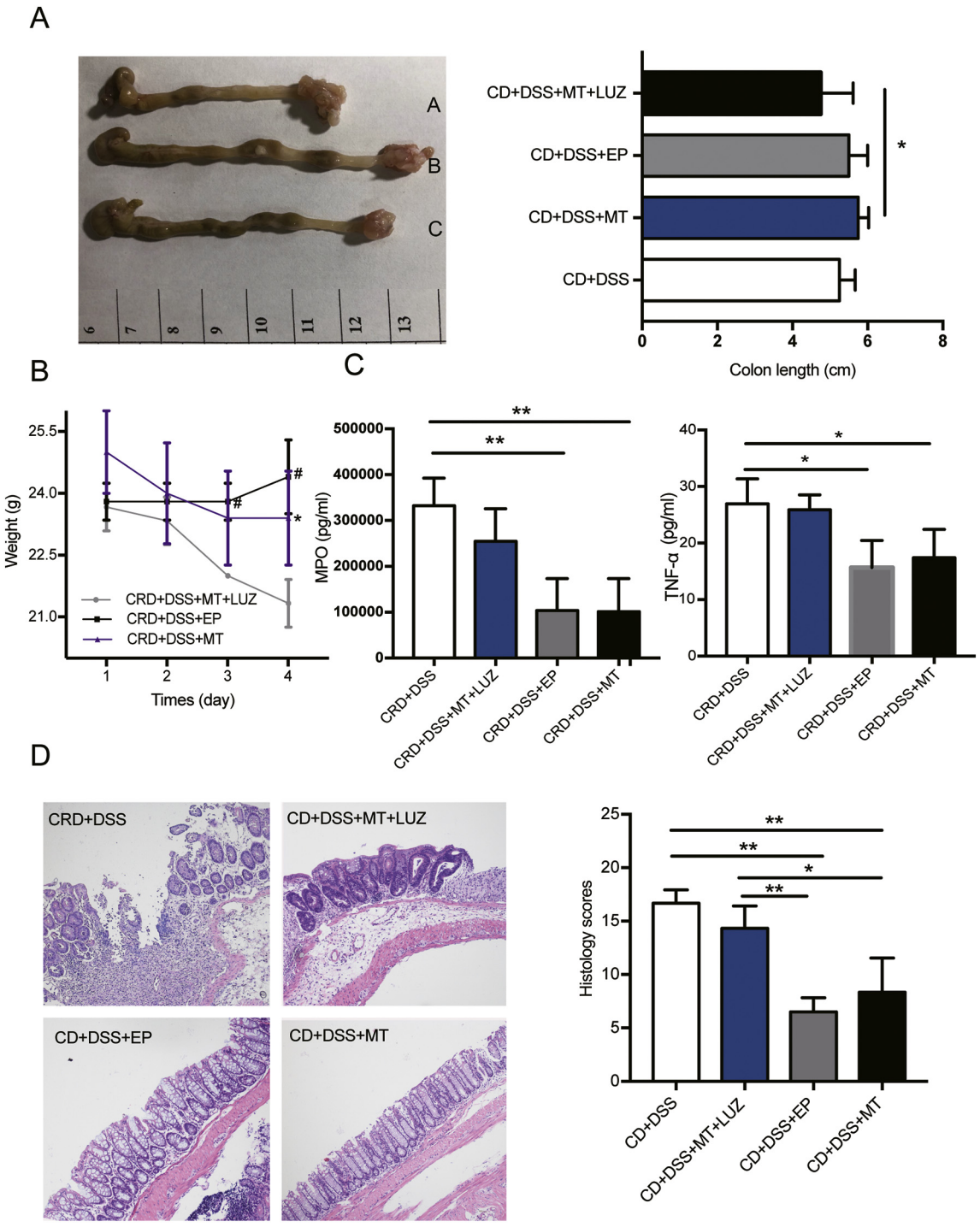

Fig. 5. Effect of MEL, LUZ or EP on CRD mice with DSS colitis. (A) Colon length of the (a) CD + DSS + MT + LUZ, (b) CD + DSS + EP, and (c) CD + DSS + MT groups, (B) body weight, (C) MPO and TNF-α levels, (D) HE staining and histological scores. All values are means ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

concentration is approximately 400 times more than that in the pineal gland [34]. The secretion is related to food intake [35], which may be influenced by the circadian rhythm [26]. Gastrointestinal melatonin participates in the motility regulation of the GI tract through its membrane receptors, including the melatonin receptor (MT), serotonin (5-HT) [36] and inhibition of nitric oxide synthase (NOS) activity or nicotinic channels [37]. Previous studies have revealed that CRD can suppress the level of melatonin, further exacerbating DSS-induced colitis, as shown in our study [11,12]. Additionally, melatonin can modulate intestinal immunoreaction by inhibiting macrophage activity, regulating proinflammatory cytokine expression and through antioxidant effects [38]. It was reported that UC patients have reduced levels of melatonin, and the levels are typically lower in active patients [39,40]. Therefore, melatonin has been studied as an adjuvant treatment for IBD both in animal trials and clinically. Some animal studies suggested that sleep deprivation and CRD aggravated inflammation in mice induced by DSS or TNBS, while melatonin significantly attenuated colitis [16,17]. Furthermore, melatonin therapy helped UC patients undergo sustain remission with the adjuvant mesalazine [39]. This refers to melatonin's capacity to reduce myeloperoxidase activity (MPO), stimulate antioxidant enzymes, and limit inflammatory cytokine levels, among which NF-κB is important to upregulate the expression of TNF-α,

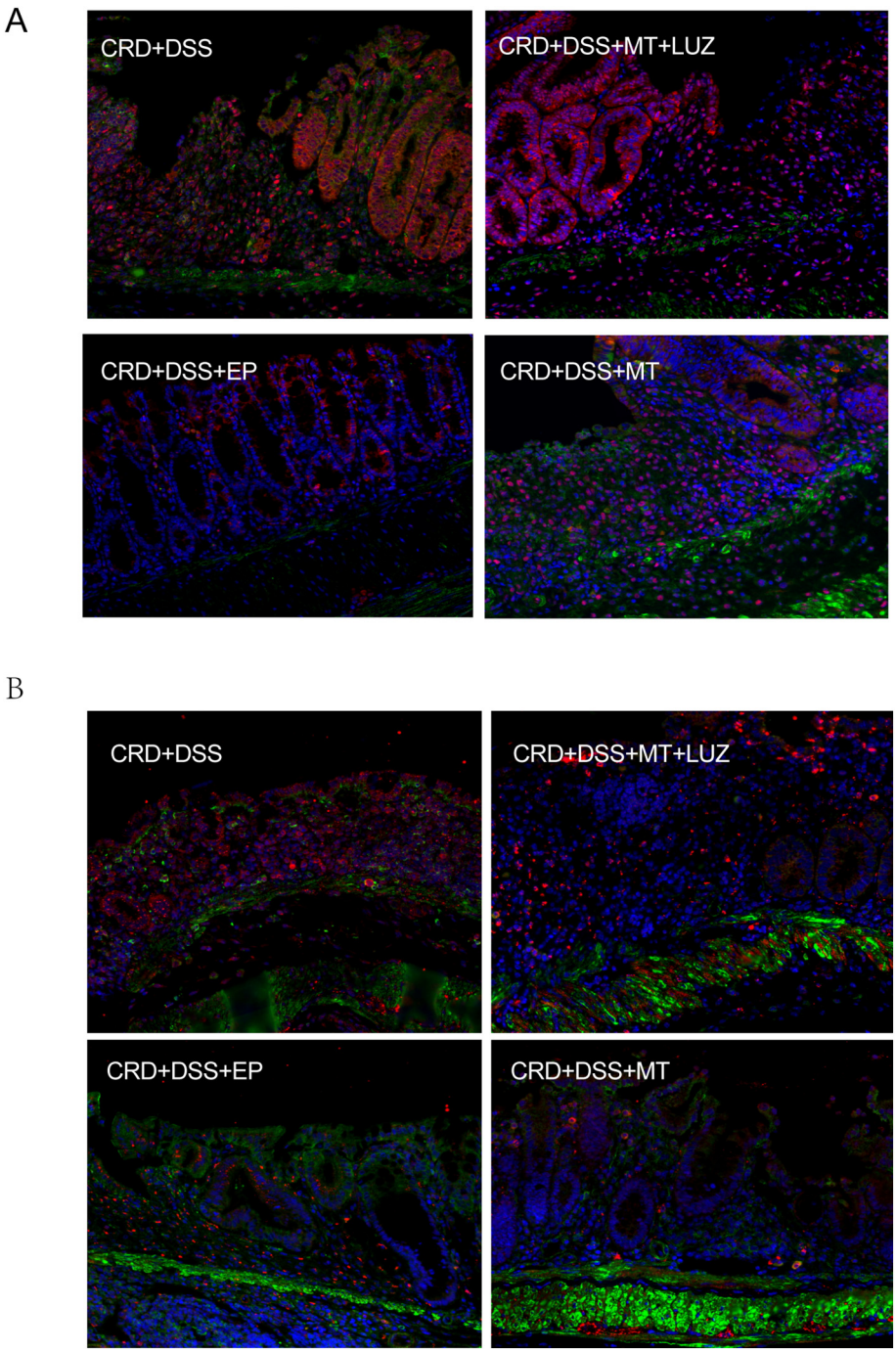

Fig. 6. Effect of MEL, LUZ or EP treatment on CRD mice with DSS-induced colitis, as evaluated by immunochemical staining performed as in Fig. 4. Melatonin and ethyl pyruvate dramatically alleviated the release of HMGB1 and TLR4 that was abolished by luzindole.

IL-1β, and COX-2 to induce inflammation [41–43].

A host of studies has demonstrated that HMGB1 has a tight relationship with IBD [44,45]. Increased intestinal and faecal HMGB1 levels were found in IL-10$^{-/-}$ mice with established colonic colitis, while ethyl pyruvate inhibited the secretion of HMGB1 to ameliorate colitis [44]. Moreover, faecal HMGB1 was confirmed to be a reliable biomarker of intestinal inflammation, identifying histological inflammation in subjects with IBD in clinical and endoscopic remission [45]. HMGB1 released by inflammatory and necrotic cells acts a DAMP molecule to induce inflammatory cascades such as the TLR4-related NF-κB signalling pathway. Numerous studies have demonstrated that HMGB1 nucleocytoplasmic translocation plays a vital role in the potential mechanism of the proinflammatory process [44,46].

We discovered that DSS-induced CRD mice were accompanied by the overexpression of HMGB1 in the cytoplasm, as well as that of TLR4 and NF-κB, in line with only DSS-induced or CRD mice. However, HMGB1 nucleocytoplasmic or intercellular and extracellular translocation, combined with TLR4 and NF-κB expression, was reversed by ethyl pyruvate, an HMGB1 antagonist (Figs. 4 and 6).

Melatonin was reported to alleviate NF-κB-induced inflammation by suppressing the HMGB1 combination with TLR4 in models of brain damage [20], sepsis-induced cardiac dysfunctionv [21] and liver ischaemia and reperfusion injury [47]. Additionally, a recent study pointed out that the protective role of melatonin in acute renal injury was closely related to the inhibition of the nucleocytoplasmic translocation of HMGB1 in renal tubular epithelial cells [48]. Likewise, we

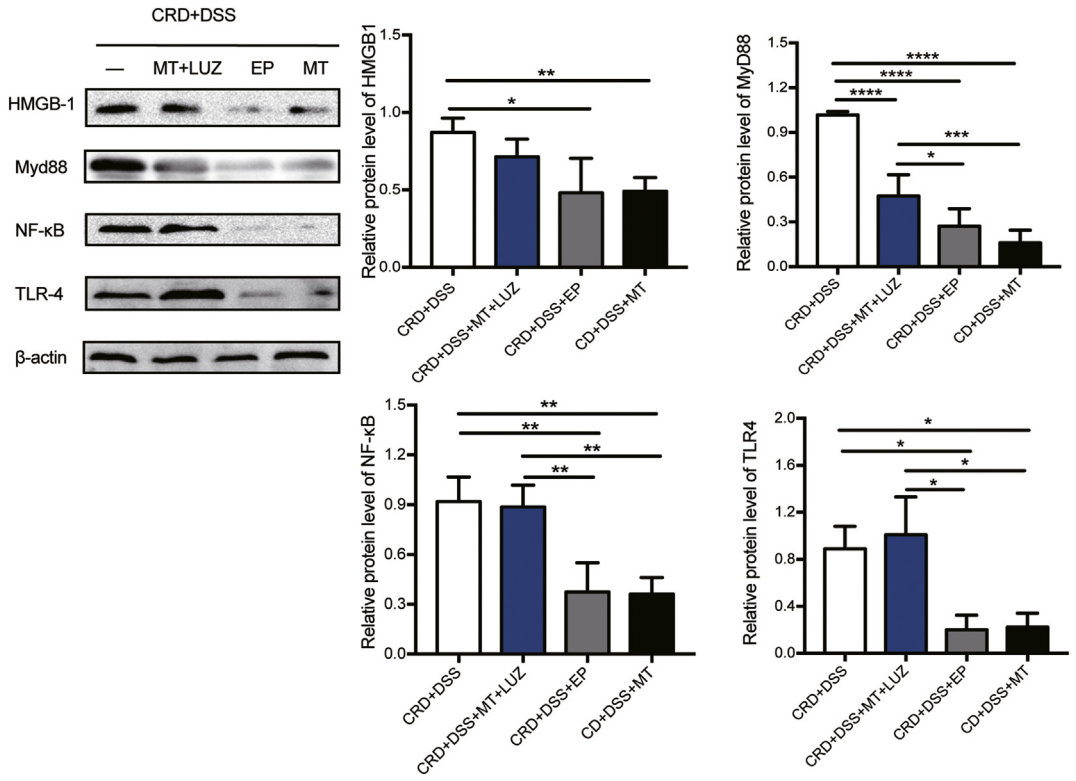

Fig. 7. Effect of Mel, LUZ or EP treatment on CRD mice with DSS-induced colitis by Western blot analysis. The protein levels of HMGB1, Myd88, NF-κB and TLR4 were downregulated by melatonin and ethyl pyruvate, while the effect was partly reversed by luzindole.

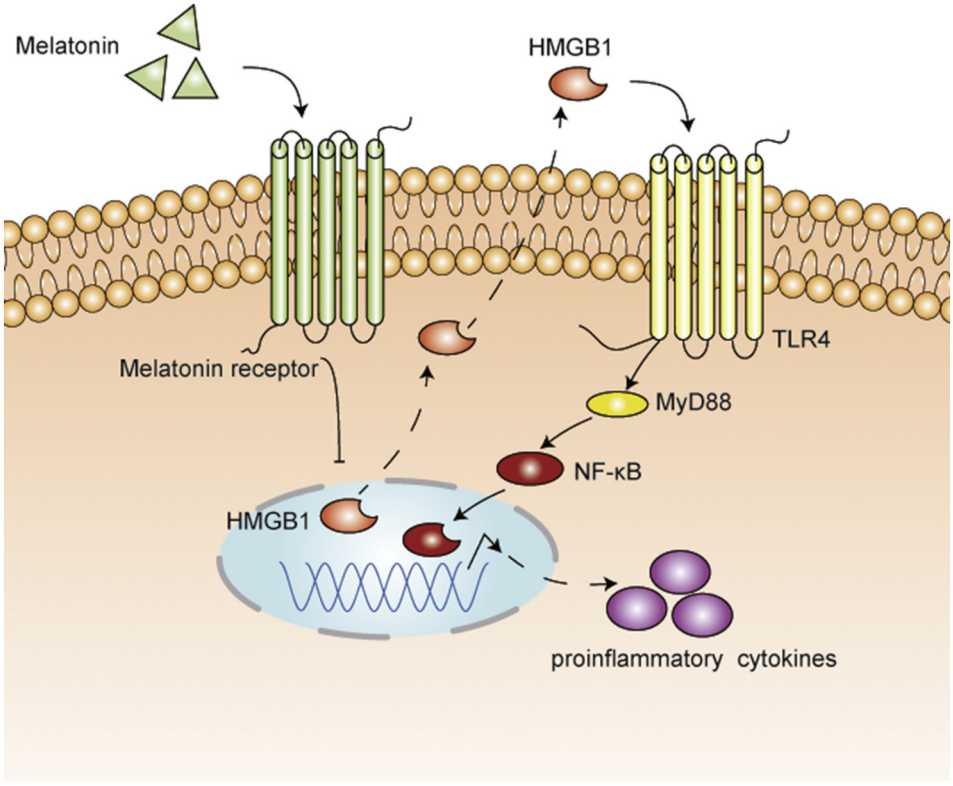

Fig. 8. Melatonin alleviates inflammation by inhibiting the distribution of HMGB1 in intestinal tissues.

observed that melatonin dramatically alleviated the levels of HMGB1 in the cytoplasm that was partly reversed by the melatonin antagonist luzindole, indicating that melatonin was involved in the location and release of HMGB1. Additionally, the decline of inflammatory mediators like TLR4 and NF-κB in the melatonin treatment group also verify the anti-inflammatory effect of melatonin (Figs. 3–7).

Accordingly, we demonstrated that CRD suppressed the level of melatonin and further aggravated DSS-induced mice by elevating the levels of HMGB1, TLR4, NF-κB, and inflammatory cytokines. The protein mechanism against DSS-induced mice with CRD may be mediated through nucleocytoplasmic or intercellular and extracellular shuttling of HMGB1 in intestinal cells (Fig. 8).

To our knowledge, this is the first study to illustrate the relationship between DSS-induced colitis in mice with CRD and an increased level of HMGB1, whose distribution and level could be influenced by melatonin.

However, further studies should be carried out to illustrate the possible cellular and molecular mechanisms of HMGB1 and melatonin. Overall, these results suggested that melatonin may be a therapeutic option beneficial to IBD patients, especially those with circadian rhythm disruption.

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Disclosures

The authors declare that there are no conflicts of interest regarding this manuscript.

Author contributions

Xi-Wen Liu finished the experiments and wrote the manuscript; Wang CD put forward the idea and edited the manuscript as the corresponding author.

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