



## Research article

## Influence of sleep disruption on inflammatory bowel disease and changes in circadian rhythm genes



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## ABSTRACT

According to clinical investigations, sleep disruption (SD) can influence the immune system and cause inflammatory bowel disease (IBD). However, the detailed effects of sleep on IBD development and progression have not been clarified. Here, we used dextran sulfate sodium (DSS) to induce colitis in mice, and then interfered with SD (day-time 8:00 a.m. to 5:00 p.m.) to explore the influence of sleep on colitis by analyzing colon length, mouse body weight, disease activity index (DAI) score, pathology detection, and infiltration of inflammatory cells with LCA immunohistochemistry analysis. Next, we detected the mRNA levels of circadian genes and related inflammatory factors, including Bmal1, CLOCK, Cry1, Cry2, Per1, Per2, Timeless, Rev-er $\alpha$ , TNF- $\alpha$ , IL-6, and IFN- $\gamma$ . Additionally, we conducted a sleep survey in IBD patients and collected colon lesion sites to detect the mRNA levels of those eight circadian genes and three inflammatory factors. We found that SD promoted the body weight decrease, increased inflammation as shown with pathological staining of the DSS animal model, and increased expression of the clock gene Cry2 in DSS-induced colitis mice. In IBD patients with active disease, the mRNA level of circadian genes Bmal1, Cry1, Cry2, and Rev-er $\alpha$  in inflammatory tissues decreased significantly compared with non-inflammatory tissues.

## 1. Introduction

Intensive stress, depression, anxiety, and many adverse psychological conditions often lead to sleep disorders. An analysis in China in 2017 showed the global prevalence of insomnia [1]. Sleep disorders have extensive physical and mental side effects and are associated with many diseases, including inflammatory bowel disease (IBD). IBD, an inflammatory disease that mainly occurs in the colon, includes two main diseases, that is, ulcerative colitis (UC) and Crohn disease (CD) [2].

Recently, the incidence and recurrence of IBD have greatly increased, and IBD is a severe chronic health problem with management and treatment challenges [2]. Therefore, it is important to understand its risk factors and then identify new treatment options.

It has been clinically recognized that IBD patients experience sleep problems and that the recurrence of IBD is in part related to sleep reduction [3]. Sleep problems among IBD patients result in reduced quality of life and increase the possibility of surgery and hospitalization [2]. A growing number of studies have paid attention to the influence of sleep on IBD. Additionally, some studies have pointed to abnormal circadian gene levels during IBD development [1].

In a mouse model of colitis, a phase shift in the sleep pattern exacerbates colitis [4]. The core circadian clock of suprachiasmatic nuclei of the anterior hypothalamus is the central oscillator. Peripheral clock genes in tissues are the peripheral oscillator and have their special functions. Sleep change may cause or be caused by circadian genes abnormalities [5]. A dextran sulfate sodium (DSS)-induced colitis model in mice shows marked changes in circadian gene expression profiles [4].

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**Table 1.** Scoring standard of Disease Activity Index (DAI). Individual scores (0–4) for percentage of weight loss, stool consistency, and blood in stool were combined to generate the DAI (0–12), which was calculated daily for each mouse.

Score	Percentage of weight loss	Stool consistency	Blood in stool
0	0	Normal	No blood
1	1%–5%	Loose stool	Presence of blood
2	5%–10%	Watery diarrhea	Presence of blood
3	10%–20%	Slimy diarrhea, little blood	Presence of blood
4	20%	Severe watery diarrhea with blood	Gross bleeding

However, there is insufficient understanding of the correlation between sleep disruption (SD), circadian gene changes, and IBD progression. In this study, we surveyed IBD patients about their sleep condition. A total of 55.8% of the patients believed that sleep disorders were greatly related to the recurrence of IBD. Therefore, it is of great clinical significance to conduct in-depth research and intervention research about the relationship between abnormal sleep and IBD. We observed that SD promoted IBD in a mouse model of DSS-induced colitis. Moreover, the mRNA levels of two circadian genes, *Bmal1* and *Cry1*, were abnormal in the colon tissues of both the mouse model and IBD patients.

## 2. Materials and methods

### 2.1. DSS-induced colitis model

Male 7-week-old C57BL/6n mice, weighing 20.0–21.0 g, were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd., and raised under consistent conditions (a 12-hour light and dark cycle, stable temperature of 24 °C ± 1 °C, and free access to food and water) for 7 days for adaptation. Then, the mice were divided into the following four groups in a random manner (n = 7 mice per group): control group under general conditions without treatment (CON group), sleep deprivation (SD) group, DSS (MP Biomedicals, LLC, Illkirch, France) group, and DSS + SD group. The DSS group was exposed to 2.0% (wt/vol) DSS dissolved in drinking water. SD was induced by a sweeping pole with an interval of 2 min in the cages during the sleep period (8:00 am to 5:00 pm) every day. The DSS + SD group was exposed to 2.0% DSS and subjected to SD induction. All animals were deprived of food and water during the period of SD. Mouse body weight, fecal occult blood testing (OBT), and disease activity index (DAI) were calculated every day. The scoring standard of DAI is shown in Table 1 [6]. On day 8, the sera of the mice were collected for biochemical analysis. The spleen and colon tissue weight of the mice was measured, and the colon length was recorded. Colon tissue was harvested for further processing. All animal experiments complied with the institutional guidelines, which were approved by Peking University Animal Ethics Committee (Beijing, China).

**Table 2.** Characteristics of inflammatory bowel disease (IBD) patients (n = 103).

	All patients	ulcerative colitis (UC) patients	Crohn disease (CD) patients
Mean age	37.5	39.0	36.0
Male: female	49:54	28:40	21:14
Number of patients in active stage of disease	32	22	10
Number of patients in steady stage of disease	71	50	21
Number of patients with symptoms	77	54	23
Number of patients without symptoms	26	14	12

**Table 3.** Mouse primer sequences for quantitative real-time PCR (qPCR).

Gene	Forward (5'–3' sequence)	Reverse (3'–5' sequence)
GAPDH	AGGTCGGTGTGAACGGATTG	TGTAGACCATGTAGTTGAGGTCA
<i>Bmal1</i>	TTTGTITGTCGTAGGATGTGACC	CGCAGTGTCCGAGGAAGATA
<i>Cry1</i>	GAGGCACTTACACGTTGGAA	GCATTCATTGAGGTGCTTCAA
<i>Cry2</i>	AGAAGGTGAAGAGGAACAGCAC	TAGATGTATCGAGAGGGGAAGC
TNF-α	CCTGTAGCCCACGTCGTAG	GGGAGTAGACAAGGTACAACCC
IL-6	TGATGGATGCTACCAAAGTGG	GTGACTCCAGCTTATCTCTTGG
IFNγ	TGAACGCTACACACTGCATC	GTTGCTGATGGCCTGATTGT

### 2.2. Serum analysis

Mouse venous blood was centrifuged at 3500 rpm for 10 min to obtain the serum for later detection. CRP, IgG, IgM, C3, and C4 levels in serum were detected by using a BS-240 multifunctional benchtop clinical chemistry analyzer (Mindray, India) in accordance with the manufacturer's protocol. C-reactive protein kit, Immunoglobulin G kit, Immunoglobulin M kit, Complement C3 kit, Complement C4 kit, and standard samples were purchased from Mindray, India.

### 2.3. Pathological analysis of the colon

After fixation with 4% polyformaldehyde for 48 h, the colon tissues of the mice were embedded in paraffin. Then, paraffin-embedded sections (5 μm) of the colon tissues were deparaffinized by xylene, and stained with hematoxylin and eosin (H&E). Five random fields from each tissue sample were recorded under a microscope.

### 2.4. Immunohistochemical analysis

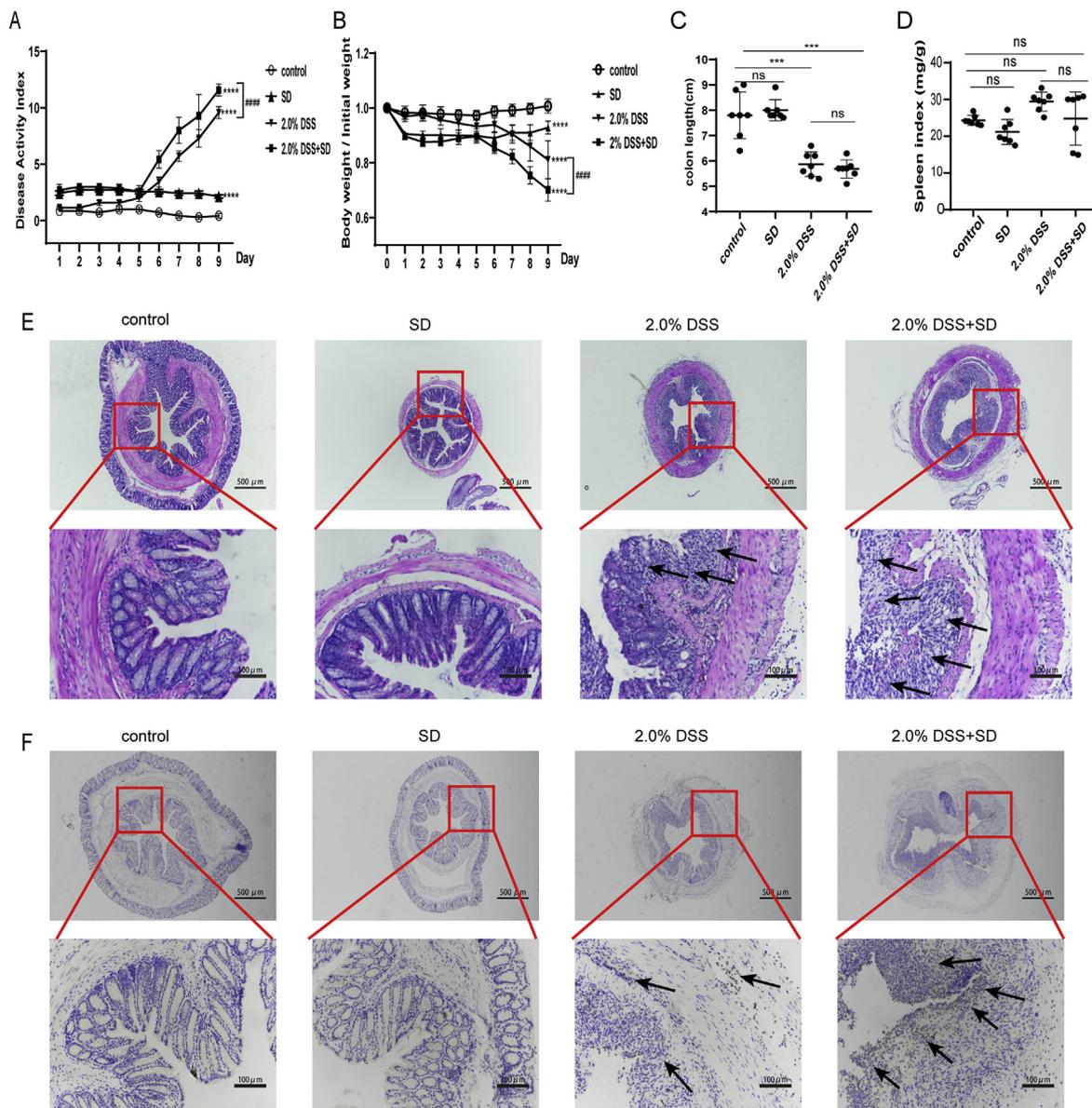
The paraffin-embedded sections (5 μm) were deparaffinized by xylene and rehydrated in graded ethanol solutions. After antigen retrieval, the sections were incubated with the antibody against leukocyte common antigen (LCA, Lot No. ab208022, Abcam) and secondary antibody. Next, the sections were treated with the DAB substrate kit (BN20341, Biorigin, Beijing, China), counterstained with hematoxylin, and viewed under a light microscope. A total of five random fields in each tissue sample were analyzed.

### 2.5. IBD patients and sleep quality questionnaire

Patients having IBD for 1–3 years were recruited (n = 103). The diagnosis of IBD was based on the clinical, endoscopic, and histological

**Table 4.** Human primer sequences for quantitative real-time PCR (qPCR).

Gene	Forward (5'–3' sequence)	Reverse (3'–5' sequence)
GAPDH	GAGTCCACTGGCGTCTTCAC	TGGTTCACACCCATGACGAA
<i>Bmal1</i>	GGATGTGACCCGAGGAAGAT	CGTCGTGTCCAGAACATAAT
<i>Cry1</i>	TTGGAAAGGAACGAGACGCAG	CGGTTGTCCACCATTTAGITT
<i>Cry2</i>	GGTGTGGAAGTAGTGACGGAG	GTAGGTCTCGTCGTTGTTCTC
Per1	CCCAGCACCACTAAGCGTAAA	TGCTGACGGCGGATCTTT
Per2	GACATGAGACCAACGAAAAGTGC	AGGCTAAAAGGTATCTGGACTCTG
CLOCK	ACGACGAGAAGCTTGGCATTG	TCCGAGAAGAGGCAGAAGG
Timeless	GAGACTTCTGCTCTGAGTTCC	CCAAGGCCACATATAATAGGT
Rev-erba	ATCGTCCGCATCAATCGCAA	CTGCTTCTCTGTTTGGGGAT
TNF-α	GGGTGTGAGAAGAGAGATGGG	GGCCAGAGGGCTGATTAGAG
IL-6	ACTCACCTCTTCAAGCAATTG	CCATCTTTGGAAGGTTGAGTTG
IL-17	CCCGGACTGTGATGGTCAAC	GCTCTTACAGTGTCTCTCC
IL-21	TTTGCTTCTTAGTTACTCACGGT	TATAAGGGGACTGGGGGCTG
IFNγ	TTTGGGTTCTCTGGCTGTT	TCCACACTCTTTTGGATGCT



**Figure 1.** Influence of sleep disruption (SD) on the development of DSS-induced mice colitis. The DSS (2.0%) animal model was used to evaluate the progression of colitis in the presence of SD. SD interference was done from 8:00 am to 5:00 pm every day. (A) Disease activity index (DAI), (B) body weight, (C) colon length, and (D) spleen weight index of IBD mice were observed and detected. (E) Evaluation of colon histology and LCA immunohistochemistry detection in the DSS-induced colitis model interfered with SD. Hematoxylin–eosin staining showed more excessive inflammation in mice exposed to SD and 2.0% DSS drinking water. The black arrow indicates the inflammatory area. (F). Infiltration of leukocytes, marked by anti-CD45, in the colon tissues was detected by immunohistochemistry and confirmed that the inflammatory cells increased in DSS-induced colitis tissues after SD interference. The black arrow shows the CD45-positive area. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , mean significant difference when compared with the control group. “ns” means that there is no significant difference. # means 2%DSS + SD compared with 2%DSS.

criteria, and the exclusion of infectious and systemic diseases. The colitis activity index was used to assess the clinical activity of the disease. Participants’ characteristics are outlined in Table 2. The Pittsburgh sleep quality index (PSQI) was used to evaluate sleep quality of IBD patients. All experimental procedures were done in strict adherence to the guidelines from the Ethics Committee of Peking University (Beijing, China).

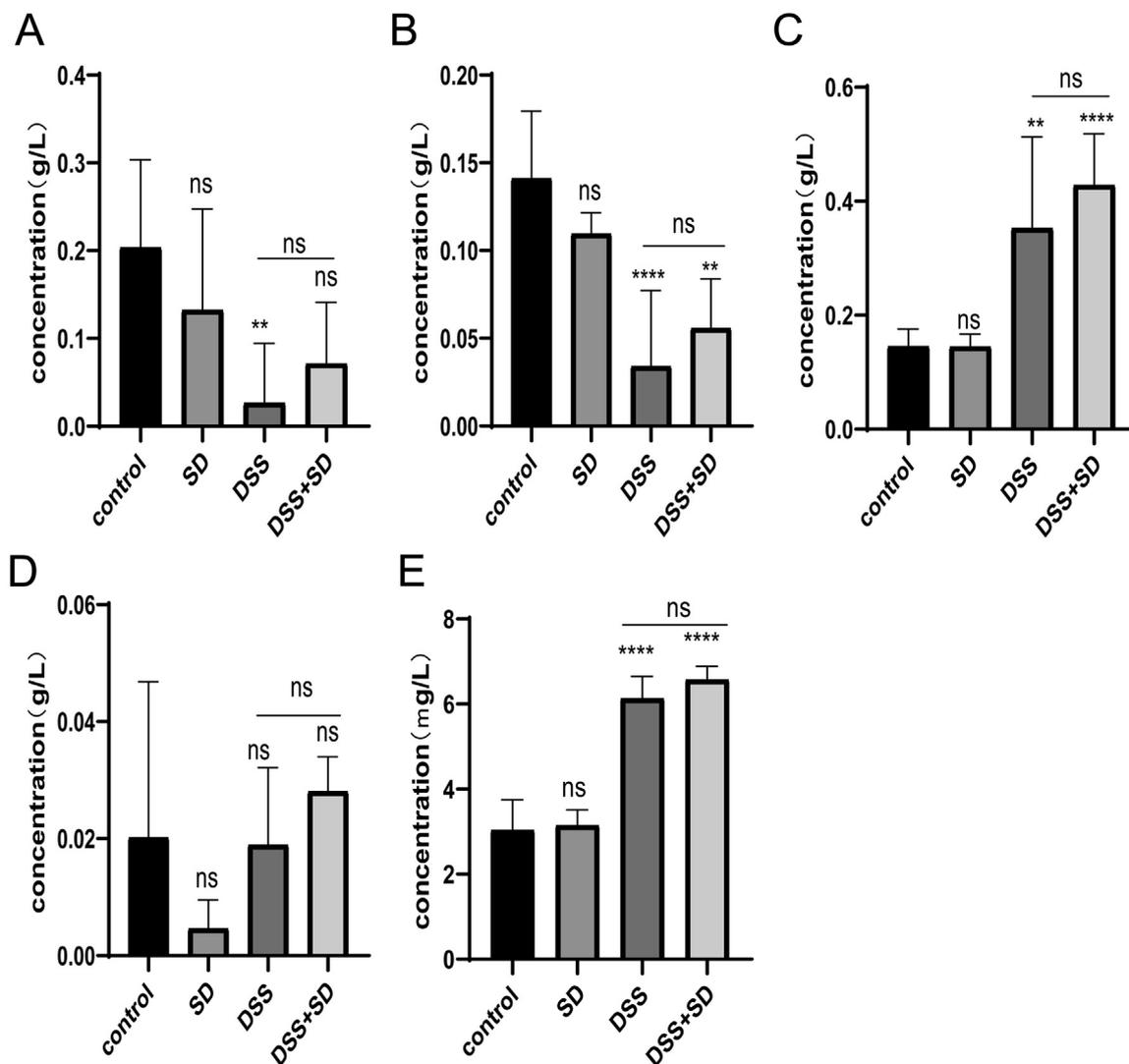
## 2.6. Preparation of mucosal samples from patients

We obtained biopsy samples from the actively inflamed mucosal sites or noninflamed colon tissues sites of IBD patients during the process of endoscopic examinations. The noninflamed tissue sites refer to nonfocal

mucosal sites observed and taken under the endoscope. After obtaining, the biopsy samples were immediately stored at  $-80^{\circ}\text{C}$ .

## 2.7. Quantitative real-time PCR

We used TRIzol reagent (Invitrogen, CA, United States) to extract total RNA from the tissue samples. Next, total RNA was reverse-transcribed into cDNA by using MonScript™ RTIII All-in-One Mix with dsDNase (MR05101M, Monadbiotech, China). We used SYBR Master Mixture (TAKARA, Dalian, China) for quantitative real-time PCR. GAPDH was used as the endogenous control, and the  $2^{-\Delta\Delta\text{Ct}}$  method was used for quantification. The primer sequences for genes of mice are listed in Table 3, and those for genes of humans are listed in Table 4.



**Figure 2.** The levels of immunoglobulin G (IgG, Figure A), immunoglobulin M (IgM, Figure B), complement 3 (C3, Figure C), complement 4 (C4, Figure D), and C-reactive protein (CRP, Figure E) in the serum of 2% DSS-induced colitis tissues after SD interference were detected. Data represent mean  $\pm$  standard deviation ( $n = 7$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared with the control group. "ns" means that there is no significant difference.

## 2.8. Statistical analysis

The data are shown as means  $\pm$  SD. Two-tailed Student's *t* test and one-way analysis of variance (ANOVA) were utilized to analyze the differences between the groups with GraphPad Prism 8.0 software. When *P* was lower than 0.05, the result was considered statistically significant.

## 3. Results

### 3.1. Effects of SD on IBD development induced by different concentrations of DSS

In the animal models, we found that SD interference from 8:00 am to 5:00 pm significantly affected the DAI score of mice fed with 2.0% DSS. The DAI score increased greatly from day 4 (Figure 1A). The mice subjected to SD and 2.0% DSS drinking water showed a significant decrease in body weight from day 6 ( $P < 0.05$ ) compared with mice that were only exposed to 2.0% DSS drinking water (Figure 1B). Hence, SD exacerbated the progression of IBD induced by DSS, but we did not find colon length reduction or spleen index changes after SD (Figure 1C and 1D).

Furthermore, pathohistological analysis showed that colon tissue was greatly damaged and inflammation was increased in mice exposed to SD

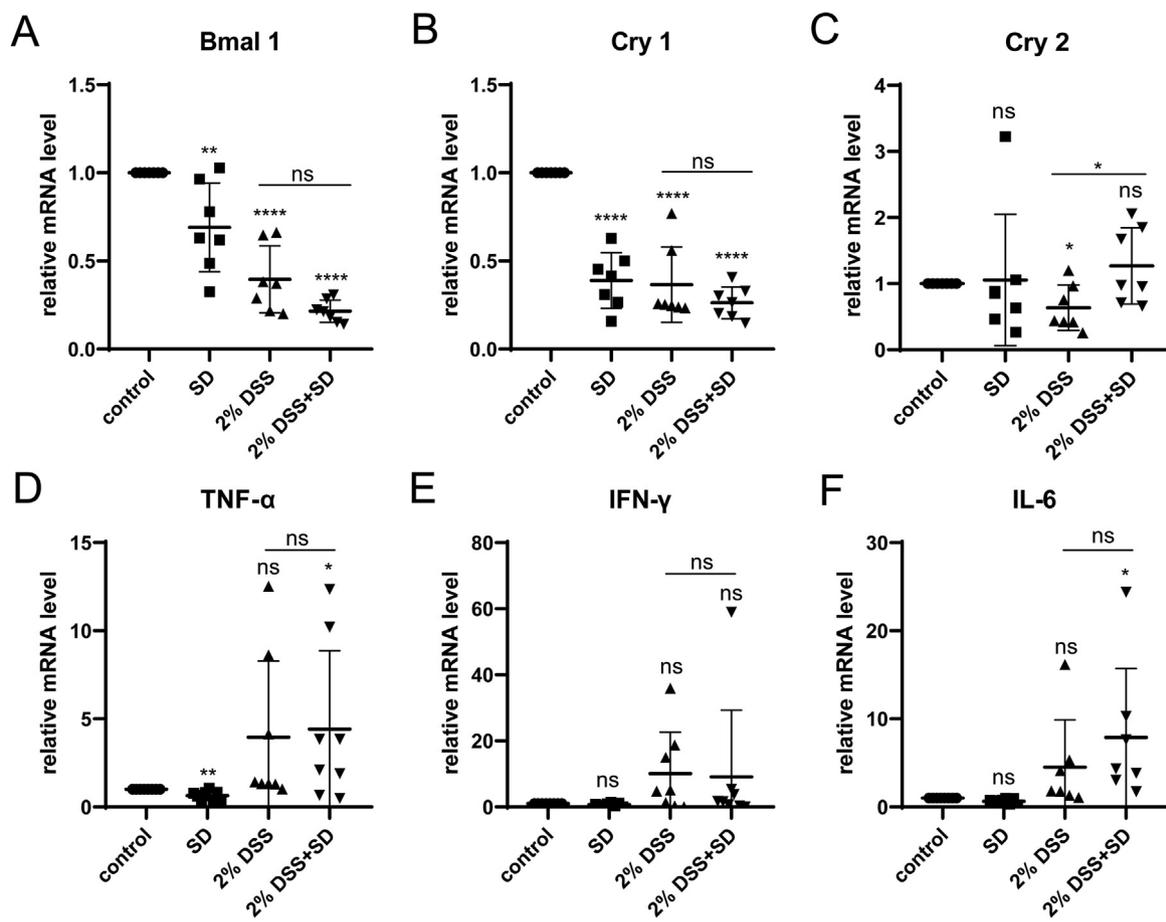
and 2.0% DSS drinking water (Figure 1E). LCA analysis confirmed that SD interference increased infiltration of the tissue by inflammatory cells (Figure 1F).

### 3.2. Effects of SD on DSS-induced serum CRP, IgG, and IgM levels

Mouse sera were collected, and CRP, IgG, IgM, C3, and C4 levels were analyzed by using the BS-240 multifunctional benchtop clinical chemistry analyzer. Compared with the DSS group, the DSS + SD group showed no significant changes in any of these indicators. However, the levels of CRP, C3, and C4 showed an insignificant tendency to higher values in the DSS + SD group compared with the DSS group (Figure 2A, B, C, D, E).

### 3.3. Changed mRNA levels of circadian clock genes and inflammatory factors in colon tissues of DSS mice after SD interference

The mRNA levels of CLOCK, Bmal1, Cry1, Cry2, Per1, Per2, Timeless, and Rev-erba were detected by RT-qPCR. We showed that Cry2 mRNA level was significantly higher ( $P < 0.05$ ) in the DSS + SD group than in the DSS group. In contrast, Bmal1 and Cry1 mRNA levels were slightly decreased in the DSS + SD group, but statistical significance was not



**Figure 3.** Detection of circadian genes and inflammatory mediators in DSS-induced colitis model interfered with SD. By using qRT-PCR and recording the cycle passing threshold (Ct), the relative mRNA levels of circadian genes and inflammatory factors in the colon mucosa tissues were detected. We utilized GAPDH as the endogenous control for data normalization. Data represent mean  $\pm$  standard deviation (n = 7). \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, \*\*\*\* $P$  < 0.0001. “ns” means that there is no significant difference.

reached (Figure 3A, B, C). Other circadian genes showed no significant changes after SD interference (data not shown).

TNF- $\alpha$ , IL-6, and IFN $\gamma$  mRNA levels were also examined. TNF- $\alpha$  and IFN $\gamma$  levels slightly increased with SD interference in DSS-induced colitis mice, but there were no significant differences compared with the DSS group (Figure 3D, E, F).

### 3.4. Sleep quality analysis in IBD patients

The PSQI was used to survey the sleep quality in 103 IBD patients. A total of 55.8% of the patients reported sleep disorders. The mean PSQI score was  $8.07 \pm 2.91$ . Patients completely without symptoms had the mean PSQI score of 6.19, whereas patients with some or severe symptoms had the mean PSQI score of 9.105, and the difference was significantly significant ( $P$  < 0.05). In IBD patients, we showed that sleep quality correlated with disease symptoms.

### 3.5. Changes in circadian clock gene and inflammatory factor mRNA levels in the tissues from IBD patients

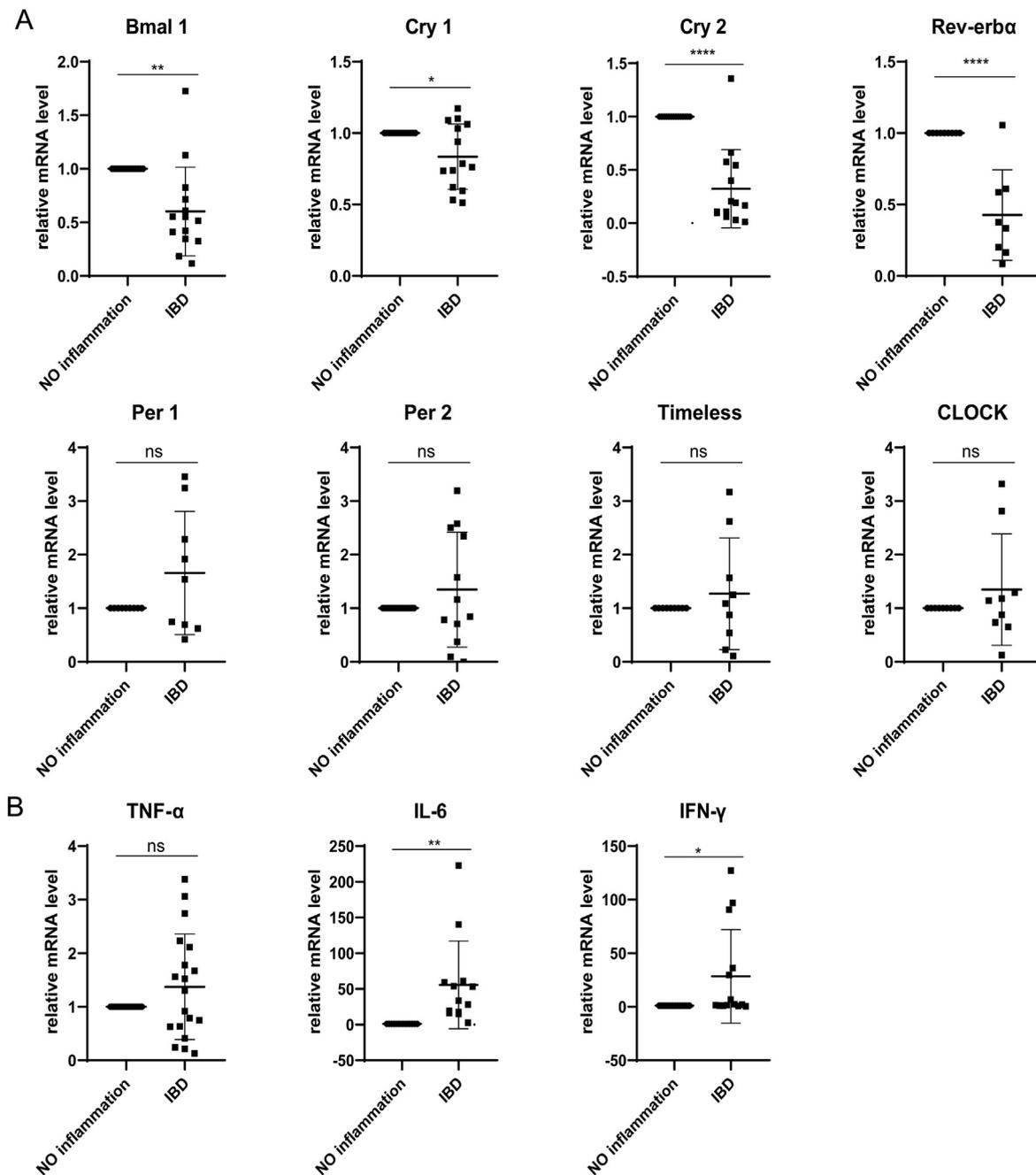
Inflamed and noninflamed colon tissues of IBD patients were collected, and the mRNA levels of CLOCK, Bmal1, Cry1, Cry2, Per1, Per2, Timeless, and Rev-erba were detected by qPCR. Bmal1, Cry1, Cry2, and Rev-erba mRNA levels were greatly reduced in inflamed tissues compared with noninflamed tissues. There were no significant differences in the mRNA levels of other circadian genes between inflamed and noninflamed tissues (Figure 4A).

TNF- $\alpha$ , IL-6, and IFN $\gamma$  mRNA levels were detected. We showed that IL-6 and IFN $\gamma$  levels were significantly higher in inflamed tissues compared with noninflamed tissues ( $P$  < 0.05, Figure 4B).

## 4. Discussion

The incidence rate of IBD is still rising all over the world. The prevalence of IBD is more than 0.3% of the whole population in several European and American countries [3, 7]. The annual incidence rate of IBD is 1.37 in Asia, and China has the highest incidence rate of IBD among Asian countries [8]. The pathogenesis of IBD has not yet been completely clarified. It likely involves complex interactions among host, microorganism, and environmental factors [9]. More than 240 genetic variants in the human genome are related to IBD and are involved in signals related to innate and adaptive immunity, defective bacterial handling, autophagy, and IL-23 and IL-10 factors [10]. Recently, there has been some progress in IBD treatment, leading to improved mucosal healing, normalization of several blood markers, and symptom disappearance. However, because of the heterogeneity of IBD disease, it is still hard to cure [11]. The recurrence of IBD brings great pain to patients and places a great burden on their families and society. Therefore, the treatment of IBD patients focuses on maintaining remission and avoiding recurrence. Identifying and avoiding recurrence factors is of great clinical significance to avoid the recurrence of IBD.

Poor objective and subjective sleep quality has been reported in IBD patients [12]. Our study also showed that 55.8% of the patients thought that their relapse was related to sleep disorders. The sleep disorders of



**Figure 4.** Detection of circadian genes (A) and inflammatory factors (B) in the colon mucosa inflammation tissues of IBD patients. qRT-PCR method was used. Data represent mean  $\pm$  standard deviation ( $n = 8\text{--}20$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , compared with noninflammatory tissues. “ns” means that there is no significant difference.

IBD patients include long sleep latency, reduced efficiency, delay, frequent sleep interruption, and high drug use rate. The decline in sleep quality may aggravate gastrointestinal symptoms, lead to the deterioration of disease, cause anxiety and depression, and seriously reduce the quality of life of these patients [13]. Thus, not only disease progression but also sleep quality and disability should be monitored and maintained in IBD management. Indeed, sleep disorders may be one of the important risk factors for IBD recurrence [3]. Within 6 months, the recurrence rate in CD patients with low sleep quality is twice as high as that in patients with normal sleep. Our survey results indicated that the recurrence rate of IBD patients with abnormal PSQI indexes was 47% at 3 months and 67% at 6 months.

Our animal study showed that SD was able to increase the inflammatory effects of 2.0% DSS on the colon. The body weight decreased

significantly in mice simultaneously exposed to SD and 2.0% DSS. Although the colon length was not reduced in the DSS + SD group, pathohistological observations documented more abundant inflammatory damage in the colon mucosa of the mice from the DSS + SD group. LCA immunohistochemical analysis revealed the presence of immune cells in the colon tissue of the DSS + SD group. Mouse serum biochemistry analysis showed that the levels of CRP, C3, and C4 had the increasing tendency after SD interference in mice exposed to 2.0% DSS. All of these results showed the influence of sleep on IBD occurrence and development. Namely, SD promoted the inflammatory response in the colon tissue and increased serum markers in mice with 2.0% DSS-induced colitis.

The sleep–wake system is strongly related to the circadian rhythm, and changes in one aspect affect the other. Sleep disorders could lead to the interruption of the biological clock and cause changes in the circadian

rhythm [14]. For example, partial SD in Syrian hamsters under constant dark conditions leads to the advancement of a large circadian rhythm for up to 4 h [15], indicating that the neurophysiological effect of SD is sufficient to reset the circadian rhythm. The formation of circadian rhythms is based on the molecular oscillation of the core clock genes [16, 17]. These "circadian clocks" are encoded by an automatically regulated transcription translation feedback loop (TTFL). These automatic regulatory feedback loops constitute circadian rhythms [18]. A series of clock control genes (CCGs) are regulated by these core clock genes or circadian clocks. Many biological processes, such as cell metabolism, cell growth, cell cycle, DNA damage repair, and the immune system, are regulated by CCGs [19]. Therefore, we detected the changes of the circadian clock genes in DSS-induced colitis mice and IBD patients to confirm the effects of sleep disorder on IBD. We found that Bmal1, Cry1, Cry2, and Rev-erba were notably less expressed in patients with active IBD, but we did not find significant changes in CLOCK, Per1, and Per2.

We confirmed that sleep disorders can promote the development of IBD in the DSS-induced animal model. Therefore, we hypothesized that circadian gene changes play an essential role in inflammation and IBD, that is, inflammation worsens in IBD and may be regulated by circadian clock genes. The human body also carries out an adaptive immune response by adjusting the distribution of immune cells and enhancing the expression of cytokines [20]. The production of a variety of cytokines has a circadian rhythm. For example, the level of IL-6 reaches its two peaks at 7:00 pm and 5:00 am [21]. During nocturnal sleep, the serum levels of IL-6 and IFN  $\gamma$  increase [22]. Clock proteins regulate the circadian oscillation of the immune system [23, 24], and the immune factors produced in turn influence the levels of circadian clock genes and affect sleep quality [25]. Therefore, it is possible that the inflammatory state and immune factor changes in IBD may be regulated by the circadian clock genes at the early stage. Moreover, the regulatory relationship may be bidirectional between the immune system and the central nervous system, which is mediated by the common shared signals, and clock genes are one of the important regulators [25].

In addition, sleep disorders occurring in IBD may also be related to immune factors, such as TNF- $\alpha$  [26]. Additionally, chronic inflammatory stimulation by TNF- $\alpha$  can last for days, weeks, or even longer, resulting in poor sleep, that is, sleep disorders. TNF- $\alpha$  can inhibit the expression of the circadian rhythm genes, such as HLF, DBP, TEF, Per1, Per2, and Per3, in fibroblasts [27]. TNF- $\alpha$  has also been shown to induce the overexpression of Bmal1 through dual calcium-dependent pathways in rheumatoid synovial cells. An increase in TNF- $\alpha$  may damage the function of the clock genes and make patients more prone to fatigue [28]. Therefore, we speculate that the change in IBD caused by sleep disorders occurs through the effects of clock genes on the immune system and is then aggravated by immune factors.

According to previous studies, the levels of clock genes in the inflammatory colon mucosa of young untreated IBD patients are lower than those in the normal colonic mucosa [5], suggesting that clock gene changes may be an early event in IBD. Other studies have shown that circadian rhythm genes are reduced in intestinal biopsies of IBD patients, especially in the ulcerative colon [4]. In IBD patients, circadian rhythm genes in almost all biopsy specimens negatively correlate with clinical and endoscopic activity scores [4]. The level of clock genes in peripheral blood mononuclear cells closely correlate with CRP levels and erythrocyte sedimentation rate (ESR) in IBD. Significant changes in the circadian rhythm gene expression profiles caused by exposure to DSS and lipopolysaccharide increase with the secretion of proinflammatory cytokines. Therefore, inflammation and circadian rhythm genes show complex two-way regulation, and both are related to the disease activity of IBD.

## Declarations

### Author contribution statement

Wang Dan: Performed the experiments; Wrote the paper.

Yin Houqing, Wang Xin, Wang Zequn, Han Mengyuan, Chen Jingjing, Bentuo Zhang, Xihua Wei, Xian Haocheng and Yang Baoxue: Contributed reagents, materials, analysis tools or data.

He Quanzhao: Analyzed and interpreted the data.

Pan Yan and Li Jun: Conceived and designed the experiments.

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### Data availability statement

Data included in article/supp. material/referenced in article.

### Declaration of interest's statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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