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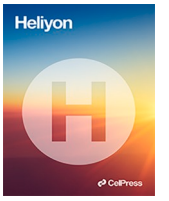

Research article

Klebsiella pneumoniae alters zebrafish circadian rhythm via inflammatory pathways and is dependent on light cues

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ABSTRACT

Klebsiella pneumoniae is an opportunistic pathogen causing severe infections. The circadian rhythm is the internal rhythm mechanism of an organism and plays an important role in coping with changes in the 24-h circadian rhythm. Disruption of the circadian rhythm can lead to immune, behavioral, mental, and other related disorders. Whether *K. pneumoniae* can disrupt the circadian rhythm after infection remains unclear. Here, we examined the effects of *K. pneumoniae* NTUH-K2044 infection on biological rhythm and inflammation in zebrafish using behavioral assays, quantitative real-time reverse transcription PCR, neutrophil and macrophage transgenic fish, and drug treatment. The results showed that *K. pneumoniae* infection decreased the motor activity of zebrafish and reduced the circadian rhythm amplitude, phase, and period. The expression of core circadian rhythm-associated genes increased under light-dark conditions, whereas they were downregulated under continuous darkness. Analysis of *Klebsiella pneumoniae*-mediated inflammation using Tg(mpx:EGFP) and Tg(mpeg:EGFP) transgenic zebrafish, expressing fluorescent neutrophils and macrophages, respectively, showed increased induction of inflammatory cells, upregulated expression of inflammatory factor genes, and stronger inflammatory responses under light-dark conditions. These effects were reversed by the anti-inflammatory drug G6PDi-1, and the expression of clock genes following *K. pneumoniae* treatment was disrupted. We determined the relationship among *K. pneumoniae*, inflammation, and the circadian rhythm, providing a theoretical reference for studying circadian rhythm disorders caused by inflammation.

1. Introduction

Klebsiella pneumoniae is a gram-negative, non-motile, facultative anaerobic bacterium that colonizes the nasal cavity and intestines of its host without causing clinical symptoms [1]. When the host immunity is unable to suppress pathogen growth, secondary infections

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including pneumonia, urinary tract infection, and sepsis, can occur [2]. Based on its virulence and contagiousness, *K. pneumoniae* is divided into classic *K. pneumoniae* and hypervirulent *K. pneumoniae* [3]. Genetic determinants of hypervirulence are typically found in large virulence plasmids and chromosomal mobile genetic elements; therefore, unique sequences on plasmids can be used to distinguish classic from hypervirulent *K. pneumoniae* [4,5]. Patients with clinical symptoms including fever, cough, sputum, leukocytosis, and pneumonia are diagnosed with *Klebsiella* respiratory tract infection [6]. Bacteria enter the human body activate the innate immune response mediated by immune cells, such as macrophages and neutrophils [7].

The circadian rhythm is an endogenous time-regulation mechanism of biological processes that enables organisms to effectively use energy and resources to forms the basis of behavior and development [8,9]. Mammals have a central pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus that helps to regulate circadian rhythms. This clock is influenced by light signals detected by the retina and synchronized with the SCN [10–12]. Given that light can directly activate gene expression [13,14], the patterns observed during regular photoperiods may be caused by circadian rhythms or may be a direct response to light. Mammalian circadian rhythms are regulated by negative feedback mechanisms involving transcription and translation. Key genes involved in this process include activators such as *CLOCK* and *BMAL1*, as well as repressors such as *PER1*, *PER2*, *CRY1*, and *CRY2* [15]. The CLOCK: BMAL1 dimer is activated during the day and drives the expression of *PER* and *CRY* by binding to the E-boxes of their promoters [16]. During night, PER and CRY are transported into the nucleus where they interact with CLOCK:BMAL1, inhibiting its activation [16,17]. When activation of CLOCK: BMAL1 is decreased, the expression of PER and CRY is reduced and the proteins are degraded, alleviating negative feedback regulation and initiating a new transcriptional cycle the next day [18,19]. Melatonin, which is synthesized and secreted by the pineal gland, is an endogenous hormone that plays an important role in regulating sleep [20]. Melatonin synthesis is catalyzed by serotonin via the product of *AANAT*, which regulates the circadian rhythm and causes melatonin levels to decrease during the day and increase at night [21,22]. Dysregulation of melatonin secretion is the primary driving force behind disrupted circadian rhythms. Additionally, misaligned circadian rhythms or timing disorders have been linked to various diseases such as psychiatric and autoimmune disorders [23].

Zebrafish are considered excellent vertebrate models because of their close evolutionary similarity to mammals, as well as their short generation time, optical transparency, and genetic manipulability. Importantly, the core components of the mammalian circadian rhythm are conserved in zebrafish. The effects of various compounds on circadian rhythms and on diseases caused by circadian rhythm disturbances have been verified in zebrafish models [24–26]. Zebrafish possess a structure similar as the mammalian SCN in the brain [27]. Unlike mammalian peripheral cells, which cannot sense light signals, all zebrafish cells are sensitive to light and temperature [10,28,29]. Similar to mammals, zebrafish exhibit circadian rhythm regulation through the negative feedback regulation of Bmal-Clock and Per-Cry [30,31]. Therefore, studies of zebrafish may provide insight into circadian rhythm mechanisms in vertebrates, including in humans.

Evidence suggests that the circadian rhythm regulates immune activation and inflammatory responses during infection, while also being regulated by immune activity [32]. Furthermore, inflammation disrupts circadian rhythms [33]. Therefore, it is important to understand whether *K. pneumoniae* infection causes circadian rhythm imbalance and the potential mechanisms underlying this effect. In this study, zebrafish were used as an animal model to investigate the effects of *K. pneumoniae* on the circadian rhythm at the behavioral and molecular levels to predict the underlying mechanisms. Our findings provide a theoretical basis for the clinical treatment of *K. pneumoniae*.

2. Methods and materials

2.1. Maintenance and husbandry of zebrafish

This study was approved by the Animal Care and Use Committee of Jiannan university (No. 2020-0015). The experimental groups included wild-type (AB strain), Tg(mpx:EGFP), and Tg(mpeg:EGFP) transgenic zebrafish. All zebrafish were maintained in a cycling culture system at 28.5 °C, and a light/dark (L/D) cycle of 14 h light and 10 h dark was established. To induce spawning, male and female zebrafish were separated in a 1:1 ratio and then placed together at 9:00 on the following day. The zebrafish laid eggs within 1 h of light exposure. Embryos were collected in 100 mm Petri dishes containing E3 solution (0.8 g/L NaCl, 0.04 g/L KCl, 0.385 mg/L Na_2HPO_4, 0.6 mg/L KH_2PO_4, 0.144 g/L $CaCl_2$, 0.246 g/L $MgSO_4$ and 0.35 g/L $NaHCO_3$) and 1 mg/L methylene blue and cultured in a light incubator. On the next day, unqualified eggs were removed, and 100 juvenile fish were retained in each dish.

2.2. Klebsiella pneumoniae acquisition and zebrafish treatment

We used a highly virulent *K. pneumoniae* strain, NTUH-K2044. The strain was obtained from our hospital and stored at −80 °C in a specialized cabinet. To activate the strain, the bacterial liquid was dipped in a sterilized inoculation ring, and lines were drawn on solid Luria-Bertani medium containing ampicillin (100 μg/mL). The medium was inverted and the bacteria were cultured at 37 °C for 20 h. Single colonies were selected and cultured in liquid Luria-Bertani medium supplemented with ampicillin at 37 °C and 180 rpm for three continuous generations. The bacteria were collected by centrifugation, and the bacterial concentration was adjusted by dilution with E3 solution to an optical density of 0.2 for experimental use. Infection of zebrafish was performed at three days post-fertilization (3 dpf) by directly replacing the zebrafish culture water with bacterial E3 solution.

2.3. Zebrafish behavioral analysis

For behavioral analysis, 3 dpf zebrafish larvae were pretreated with *K. pneumoniae*. Juvenile fish (5 dpf; n = 24 per group) were placed individually in the wells of a 48-well plate. The control group was treated with E3 solution, and the experimental group was treated with NTUH-K2044. A behavioral analyzer (Viewpoint Life Sciences, Inc., Auvergne-Rhone-Alpes, France) was used to automatically monitor the active movements of the zebrafish. The activity of juvenile fish was monitored and recorded for 3 consecutive days at a constant temperature of 28.5 °C, with a 14 h/10 h L/D cycle or in continuous darkness (D/D). ViewPoint Life Sciences, Inc. (Zebralab 3.10) software was used to record the total moving distance of the larvae per minute, which was converted to the total moving distance at intervals of 10 min. The average speed was calculated based on the swimming time of the juvenile fish. Microsoft Excel software (Redmond, WA, USA) was used to analyze the data. The phase, period, and amplitude of the zebrafish movements were analyzed using the JTK cycle method.

2.4. Quantitative real-time reverse transcription PCR

To investigate gene expression patterns in response to different light conditions, 5 dpf zebrafish (n = 30/group) were collected every 4 h for 24-h under either DD or a L/D cycle. Total RNA was extracted from each sample using a kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After DNase digestion, cDNA was synthesized from 1 μg RNA. Using β-actin as the internal reference gene, target genes were quantitatively analyzed using a LightCycler® 96 Instrument (Roche, Basel, Switzerland) and SYBR® Premix Ex Taq™ (Takara Bio, Inc., Shiga, Japan) kit. Each experiment was performed in triplicate, and relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. The primers used in this study are listed in Supplementary Table 1.

2.5. Imaging analysis

Transgenic zebrafish embryos from both the experimental and control groups were anesthetized using 0.03 % tricaine and placed on slides containing a 0.05 % agarose/E3 solution. An inverted fluorescence microscope (Leica DMI3000B, Wetzlar, Germany) was used to observe the recruitment of labeled neutrophils (Tg(mpx:EGFP)) and macrophages (Tg(mpeg:EGFP)) by adjusting the focal area to the standard green fluorescent protein (GFP) channel. Uninfected zebrafish were used as controls. The fluorescence setting was unified, and images of the juvenile fish were captured and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) to determine the numbers of neutrophils and macrophages.

2.6. Treatment with anti-inflammatory drugs

Tg(mpx:EGFP) and Tg(mpeg:EGFP) transgenic zebrafish were infected with NTUH-K2044 for 48 hpf, followed by treatment with the anti-inflammatory drug G6PDi-1. Zebrafish cultured in E3 solution were used as the control group. We also evaluated NTUH-K2044 infection and NTUH-K2044+G6PDi-1 treatment groups. G6PDi was treated at a concentration of 100 nM. Water in the control and treatment groups was exchanged once per day. The recruitment of neutrophils and macrophages, as well as the expression of genes associated with inflammation and the circadian rhythm, were evaluated in different groups of zebrafish at 5 dpf.

2.7. Data analysis

All data are expressed as the mean ± standard deviation. Statistical analysis was performed using Student's *t*-test to detect differences in the mean values between the control and treated groups. The circadian rhythm was analyzed using the online software biodare2 (https://biodare2.ed.ac.uk/). Graphs were drawn using GraphPad Prism Software (GraphPad Software, San Diego, CA, USA). All experiments were performed in triplicate. Statistical significance was set at $P < 0.05$.

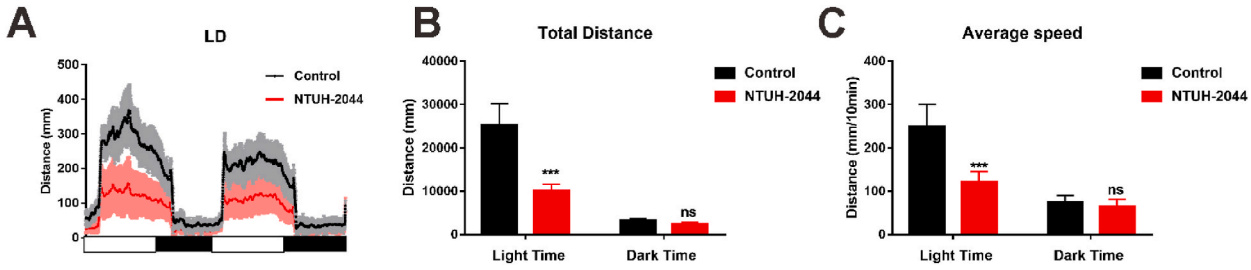

Fig. 1. Locomotor ability of zebrafish exposed to *Klebsiella pneumoniae* decreased under 14 h/10 h light-dark (L/D) conditions. (A) Locomotor ability of juvenile fish in the control and NTUH-2044 groups under L/D conditions. (B) Total travel distance of juvenile zebrafish in light and dark time periods in the control and NTUH-2044 groups. (C) Average swimming speed of juvenile zebrafish in light and dark time periods in the control and NTUH-2044 groups. Each experiment was repeated in triplicate. Data are expressed as the mean ± standard deviation (SD). Data were analyzed using Student's *t*-test. (***$P < 0.001$, ns: not significant).

3. Results

3.1. Klebsiella pneumoniae infection decreased the motor activity of zebrafish

To investigate whether *K. pneumoniae* infection affects the motor activity of zebrafish, behavioral analysis of 5 dpf juvenile zebrafish exposed to the highly virulent NTUH-K2044 *K. pneumoniae* strain was performed under 14 h/10 h L/D conditions. The results showed that compared with that of the control group, the swimming distance of zebrafish in the NTUH-K2044-infected group was significantly reduced during the daytime (Fig. 1A and B). Although the exercise ability of the NTUH-K2044-infected group was reduced compared with that of the control group at night, the difference was not significant (Fig. 1A and B). Further analysis of the swimming speed showed that compared with the control group, the average speed of NTUH-K2044-infected zebrafish was decreased significantly during the day, whereas there was no significant change at night (Fig. 1C). These data indicate that *K. pneumoniae* exposure significantly reduces the motor activity of zebrafish during the day.

3.2. Klebsiella pneumoniae alters zebrafish circadian rhythms

To explore whether *K. pneumoniae* exposure affects zebrafish behavioral rhythms, 8 dpf juvenile zebrafish from the control and NTUH-K2044-exposed groups were subjected to behavioral analysis under D/D conditions (Fig. 2A). Compared with that in the control group, the amplitude of zebrafish in the NTUH-K2044 group was significantly reduced (Fig. 2B), the period was significantly shortened (Fig. 2C), and rhythmic behavior was significantly advanced (Fig. 2D). Based on these results, treatment with *K. pneumoniae* may alter the circadian rhythm of zebrafish.

3.3. Klebsiella pneumoniae exposure affects the expression of circadian rhythm genes in zebrafish

To explore the mechanism behind *K. pneumoniae*-mediated regulation of circadian rhythms in zebrafish, we detected the expression of genes related to the circadian rhythm in 5 dpf zebrafish within 24 h using quantitative real-time reverse transcription (qRT)-PCR under L/D and D/D conditions. The results showed that under L/D conditions, the expression level of *bmal1b* was higher in the control group at Zeitgeber time 8 (ZT8) and higher in the NTUH-K2044 group at ZT12 and ZT20 (Fig. 3A). The expression level of *clock1a* was higher in the NTUH-K2044 group at ZT20 and in the control group at ZT24. There was no significant difference between groups at the other evaluated time points (Fig. 3A). Except for that at ZT24, the expression level of *cry1aa* in the NTUH-K2044 group was higher than that in the control group (Fig. 3A). The expression level of *per1b* in the NTUH-K2044 group was higher than that in the control group at ZT16 and ZT20, with no significant difference at the other time points (Fig. 3A). Except for that at ZT24, the expression level of *per2* in the NTUH-K2044 group was higher than that in the control group (Fig. 3A). The expression level of *aanat2* was significantly higher in the NTUH-K2044 group than in the control group throughout the ZT cycle (Fig. 3A). The expression level of *bmal1b* was significantly lower in the NTUH-K2044 group than in the control group during a circadian time (CT) cycle under D/D conditions (Fig. 3B). The expression of *clock1a* was significantly higher in the NTUH-K2044 group than in the control group during the CT cycle (Fig. 3 B). The expression levels of *cry1aa*, *per2*, *per1b*, and *aanat2* were lower in the NTUH-K2044 group than in the control group during the CT cycle (Fig. 3B). The expression levels of *bmal1a*, *clock1b*, *per1a*, and *cry1ab* were higher in the NTUH-K2044 group during the ZT cycle and higher in the control group during the CT cycle (Supplementary Fig. 1). Except for *clock1a*, light significantly increased the expression of circadian rhythm genes in the NTUH-K2044 group, whereas continuous darkness downregulated the expression of circadian rhythm

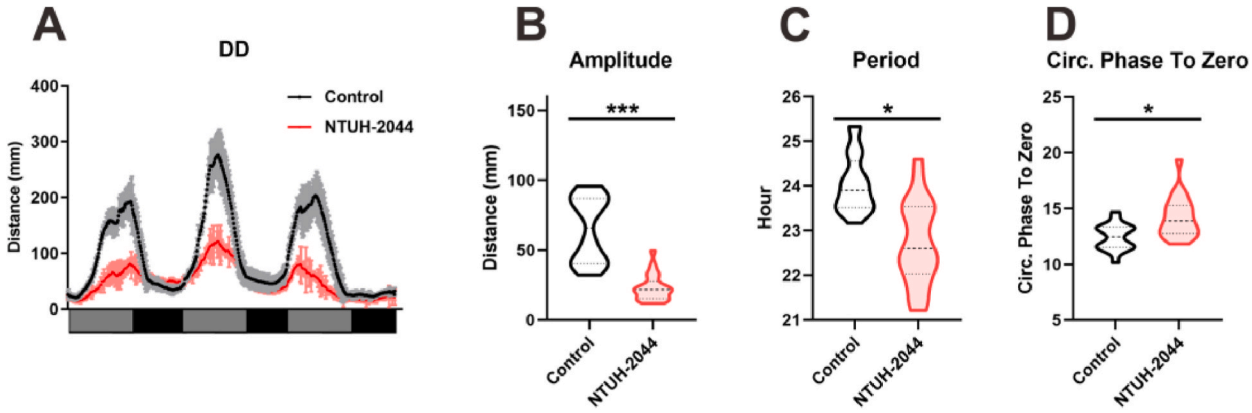

Fig. 2. Changes in behavior of *Klebsiella pneumoniae*-infected zebrafish under continuous darkness (D/D) conditions. (A) Behavioral rhythm analysis of juvenile fish in the control and NTUH-2044 groups under D/D conditions. (B) Amplitude comparison of juvenile zebrafish in the control and NTUH-2044 groups under D/D conditions. (C) Period comparison of juvenile zebrafish in the control and NTUH-2044 groups under D/D conditions. (D) Phase comparison of juvenile zebrafish in the control and NTUH-2044 groups under D/D conditions. Each experiment was repeated three times. Data are expressed as the mean ± standard deviation (SD). Data were analyzed using Student's *t*-test. (*P < 0.05, **P < 0.01, ***P < 0.001).

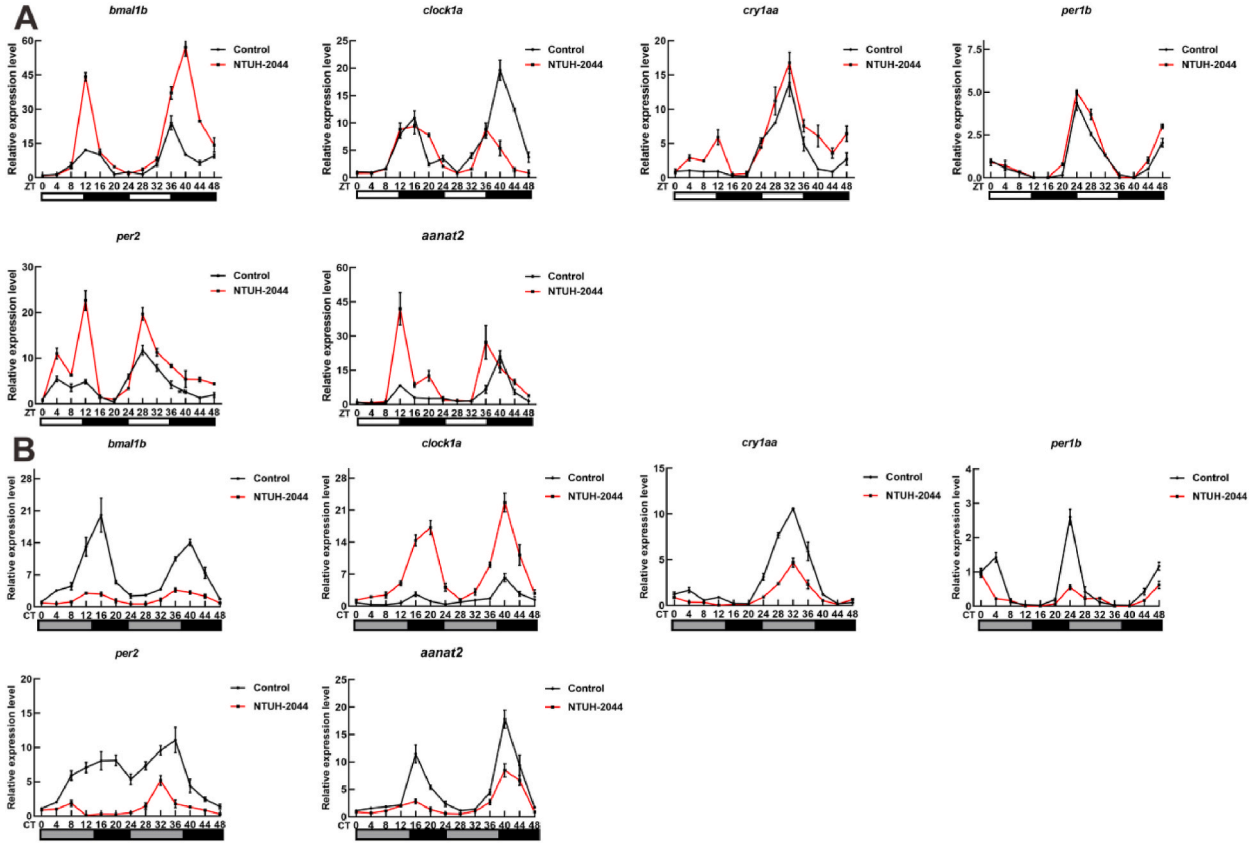

Fig. 3. Expression of core circadian rhythm genes in *Klebsiella pneumoniae*-infected zebrafish. Expression levels of (A) *bmal1b*, *clock1a*, *cry1aa*, *per1b*, *per2*, and *aanat2* in the control and NTUH-2044 groups under light/dark (L/D) conditions. Expression levels of (B) *bmal1b*, *clock1a*, *cry1aa*, *per2*, *per1b*, and *aanat2* in the control and NTUH-2044 treatment groups under D/D conditions. Each experiment was repeated three times. Data are expressed as the mean ± standard deviation (SD). Data were analyzed using Student's *t*-test. (*P < 0.05, **P < 0.01, ***P < 0.001).

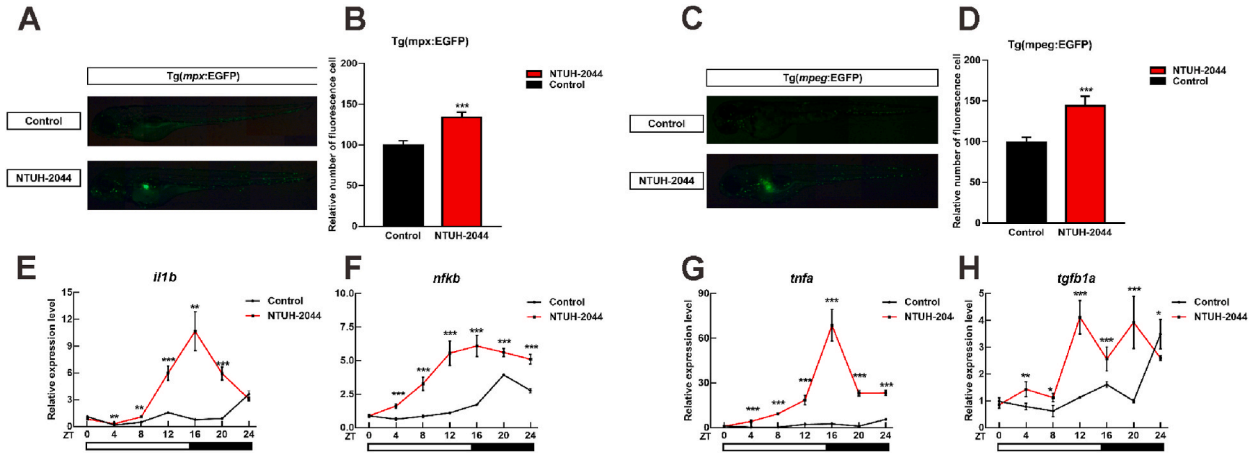

Fig. 4. Inflammatory cell and gene expression levels in zebrafish infected with *Klebsiella pneumoniae* and treated under light/dark (L/D) conditions. (A) Neutrophil recruitment of Tg(mpx:EGFP) transgenic zebrafish in the control and NTUH-2044 treatment groups under L/D conditions. (B) Number of neutrophils in Tg(mpx:EGFP) transgenic zebrafish compared with those in the control and NTUH-2044 groups under L/D conditions. (C) Macrophage recruitment of Tg(mpeg:EGFP) transgenic zebrafish in the control and NTUH-2044 groups under L/D conditions. (D) Number of macrophages in Tg(mpeg:EGFP) transgenic zebrafish compared with those in the control and NTUH-2044 groups under L/D conditions. qRT-PCR analysis of inflammation-related genes *il1b* (E), *nfkb* (F), *tnfa* (G), and *tgfb1a* (H) in the control and NTUH-2044 groups under L/D conditions. Each experiment was repeated three times. Data are expressed as the mean ± standard deviation (SD). Data were analyzed using Student's *t*-test. (ANOVA) (*P < 0.05, **P < 0.01, ***P < 0.001).

genes in the NTUH-K2044 group (Fig. 3B). We used ELISH to detect changes in melatonin levels after *K. pneumoniae* infection under LD conditions. The results revealed a significant increase in melatonin levels after treatment with *K. pneumoniae* (Supplementary Fig. 2). Changes in the biological rhythms of *K. pneumoniae* after treatment were evaluated using online analysis software; we found that *K. pneumoniae* disrupted biological rhythms under LD and DD conditions (Supplementary Tables 1 and 2). These results indicate that *K. pneumoniae* interferes with the expression of circadian rhythm genes in juvenile zebrafish and that light plays an important role in this mechanism.

3.4. *Klebsiella pneumoniae* exposure increased inflammation in zebrafish

To understand the effect of *K. pneumoniae* infection on inflammation, we examined the recruitment of inflammatory cells and expression of related genes under L/D and D/D conditions. The results demonstrated that under L/D conditions, Tg(mpx:EGFP) transgenic zebrafish larvae infected with NTUH-K2044 exhibited significantly higher GFP intensities than those in the control group (Fig. 4A), indicating increased neutrophil activation and a significantly larger number of neutrophils than those in the control group (Fig. 4B). Similarly, NTUH-K2044 exposure increased the number of macrophages in zebrafish (Fig. 4C and D). However, under D/D conditions, the numbers of neutrophils and macrophages in zebrafish infected with NTUH-K2044 did not significantly differ from those in the control group (Supplementary Figs. 3A–D). Quantitative analysis of inflammation-related genes within a 24-h window showed that the expression levels of *il1b*, *nfkb*, *tnfa*, and *tgfb1a* were significantly upregulated in the NTUH-K2044 group than in the control group during the ZT cycle (Fig. 4E–H). The expression levels of *nfkb* and *tgfb1a* were significantly downregulated in the NTUH-K2044 group during CT cycle (Supplementary Figs. 3F and H). The expression level of *il1b* was lower at CT0 and CT12 than that in the control group and higher in the NTUH-K2044 group at the other time points evaluated (Supplementary Fig. 3E). During both the CT and ZT cycles, the expression level of *tnfa* was higher in the NTUH-K2044 group than in the control group; however, the increase in *tnfa* expression was significantly greater under L/D conditions (Fig. 4G, Supplementary Fig. 3G). These data show that *K. pneumoniae* treatment significantly increased inflammation in zebrafish and likely induced inflammation under light exposure.

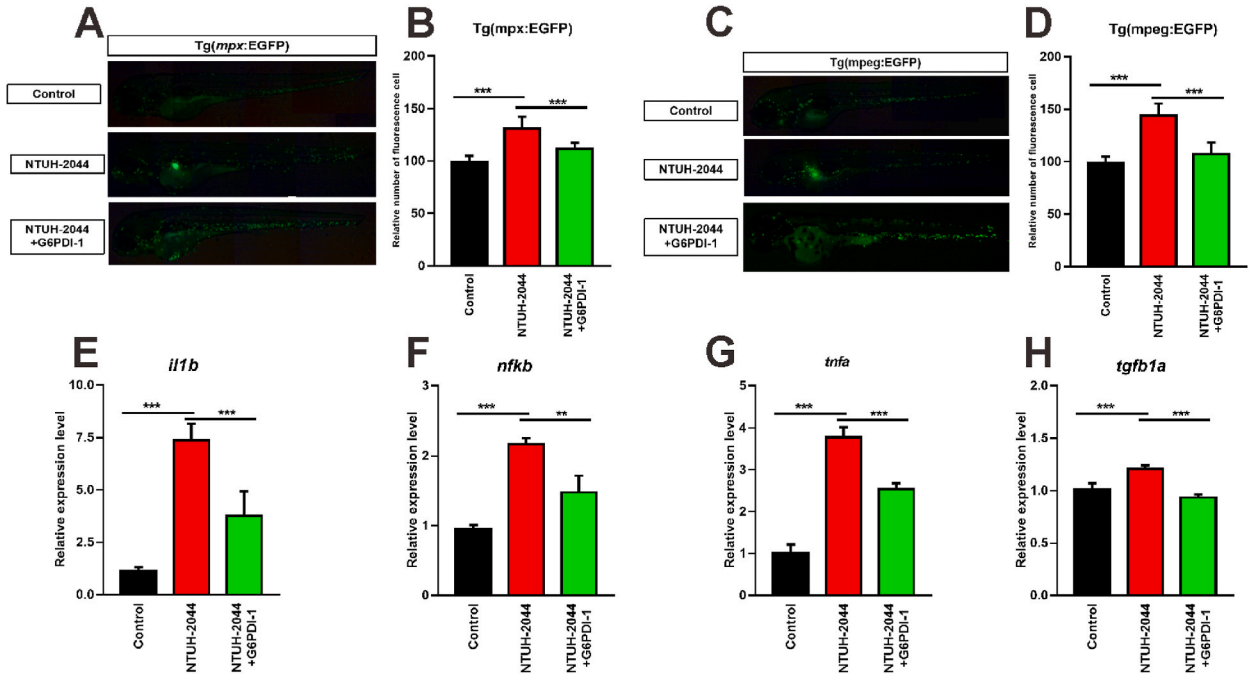

Fig. 5. Anti-inflammatory drugs reduce *Klebsiella pneumoniae*-induced inflammation. Zebrafish were infected with NTUH-K2044 for 48 hpf, followed by treatment with the anti-inflammatory drug G6PDi-1, and inflammation was evaluated at 5 days post-fertilization (dpf). (A) Neutrophil recruitment in Tg(mpx:EGFP) transgenic zebrafish in the control, NTUH-2044, and NTUH-2044+G6PDi-1 treatment groups. (B) Number of neutrophils in Tg(mpx:EGFP) transgenic zebrafish in the control, NTUH-2044, and NTUH-2044+G6PDi-1 treatment groups. (C) Macrophage recruitment of Tg(mpx:EGFP) transgenic zebrafish in the control, NTUH-2044, and NTUH-2044+G6PDi-1 treatment groups. (D) Number of macrophages in Tg(mpx:EGFP) transgenic zebrafish in the control, NTUH-2044, and NTUH-2044+G6PDi-1 treatment groups. qRT-PCR analysis of inflammation-related genes *il1b* (E), *nfkb* (F), *tnfα* (G), and *tgfb1a* (H) in the control, NTUH-2044, and NTUH-2044+G6PDi-1 treatment groups. Each experiment was performed in triplicate. Data are expressed as the mean ± standard deviation (SD). Data were analyzed by Student's *t*-test.(ANOVA) (**P < 0.01,***P < 0.001).

3.5. Anti-inflammatory drugs reduce K. pneumoniae-induced inflammation

G6PDi-1 is a potent G6PD inhibitor that depletes NADPH and reduces inflammatory cytokine production [34]. To verify whether G6PDi-1 can reduce inflammation induced by *K. pneumoniae* infection, we examined NTUH-K2044-infected Tg(mpx:EGFP) and Tg (mpeg:EGFP) transgenic zebrafish following treatment with the anti-inflammatory drug G6PDi-1. Compared with that in the control group, the recruitment of neutrophils and macrophages decreased after G6PDi-1 treatment (Fig. 5A,C). Statistical analysis revealed a significant decrease in the number of neutrophils and macrophages (Fig. 5B,D). qRT-PCR analysis showed that G6Pdi-1 partially reduced the upregulation of inflammation-related genes induced by NTUH-K2044 exposure and significantly reduced the expression of *il1b*, *nfkb*, *tnfa*, and *tgfb1a* (Fig. 5E–H). Therefore, G6Pdi-1 can reduce inflammation induced by *K. pneumoniae*.

3.6. Anti-inflammatory drug treatment rescues K. pneumoniae-mediated dysregulation of circadian rhythm gene expression

To explore whether the observed changes in the circadian rhythm could be rescued by reducing inflammation, we examined the expression of clock-related genes in NTUH-K2044-infected zebrafish following treatment with the anti-inflammatory agent G6Pdi-1. The results showed that NTUH-K2044 treatment significantly increased expression of the zebrafish circadian rhythm genes *per2*, *cry1ab*, *cry1aa*, and *clock1a*, whereas their expression levels decreased after addition of G6Pdi-1 (Fig. 6A–D). These results suggest that *K. pneumoniae* affects the zebrafish circadian rhythm through inflammatory pathways and that the anti-inflammatory agent G6Pdi-1 can reduce the expression of circadian rhythm genes by reducing inflammation.

4. Discussion

Circadian rhythms enable organisms to anticipate changes in their daily environment and to regulate many physiological and behavioral processes. Therefore, disruptions in the external environment can pose significant health risks. *Klebsiella pneumoniae* is an important opportunistic pathogen associated with iatrogenic infections. To understand the effects of *K. pneumoniae* infection on the circadian rhythm of organisms, a highly virulent NTUH-K2044 strain was used to infect juvenile zebrafish. The results showed that *K. pneumoniae* infection significantly decreased motor activity, increased inflammatory phenotypes, and altered circadian rhythms. Additionally, *K. pneumoniae* interfered with the expression of inflammatory genes and core genes involved in the circadian rhythm, which was relieved after treatment with an anti-inflammatory drug. Therefore, *K. pneumoniae* may affect the zebrafish circadian rhythm through inflammation, and light may play an important role in this process. These results suggest a mechanism through which *K. pneumoniae* dysregulates circadian rhythms and provide insight into the relationship between disease, inflammation, and the circadian rhythm.

Klebsiella pneumoniae causes circadian rhythm disorders in zebrafish. The circadian rhythm controls many physiological processes and a normal rhythm plays an important role in maintaining the normal activities of organisms [35]. In this study, treatment with *K. pneumoniae* significantly reduced the period, phase, and amplitude of zebrafish behavior, as well as the behavioral rhythm of zebrafish, and disrupted the expression of core circadian rhythm genes. Circadian disorders can be induced by numerous internal and external factors such as hormones, stress, and environmental compounds [33,36]. Recent studies have shown that populations of intestinal microbes in mammals exhibit distinct rhythmicity and synchronize with the host circadian rhythm via the gut-SCN axis [37]. *Klebsiella pneumoniae* is an intestinal-colonizing bacterium that invades intestinal epithelial cells [38]. Therefore, *K. pneumoniae* may disrupt the host circadian rhythm through the gut-SCN axis.

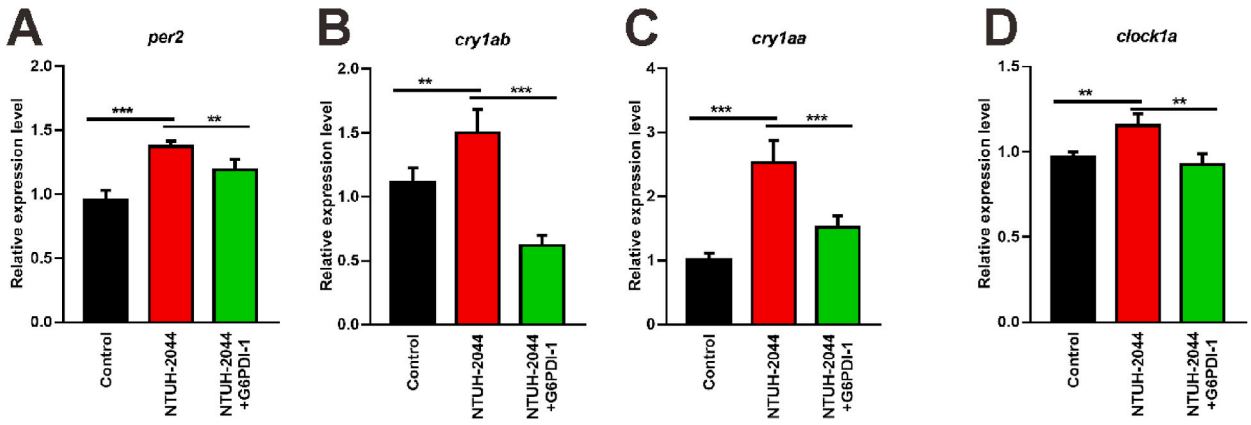

Fig. 6. Anti-inflammatory drug rescue of *Klebsiella pneumoniae*-disrupted circadian rhythm genes. Zebrafish were infected with NTUH-K2044 for 48 hpf, followed by treatment with the anti-inflammatory drug G6PDi-1, and the expression of genes associated with circadian rhythm were evaluated in different groups of zebrafish at 5 dpf. qRT-PCR analysis of zebrafish clock genes *per2* (A), *cry1ab* (B), *cry1aa* (C), and *clock1a* (D) in the control, NTUH-2044, and NTUH-2044+G6PDi-1 treatment groups. Each experiment was performed in triplicate. Data are expressed as the mean ± standard deviation (SD). Data were analyzed using Student's *t*-test. (**P < 0.01, ***P < 0.001).

We also found that *K. pneumoniae* exposure significantly increased the expression of *aanat2*, which encodes the rate-limiting enzyme in melatonin synthesis and directly regulates the circadian rhythm [39]. The expression of *aanat2* was significantly increased under L/D conditions, which may have been caused by increased expression of *bmal1b* and *clock1a* after infection with *K. pneumoniae* [40]. Previous studies have demonstrated that several microbes can disrupt melatonin synthesis [41]. Our results show that melatonin secretion was increased after *K. pneumoniae* infection. Melatonin is an important hormone with higher secretion at night than during the day in both humans and zebrafish. In a previous study, melatonin also significantly reduced inflammation, revealing an important link between the biological clock and inflammation. *Klebsiella pneumoniae* infection advanced the phase of zebrafish and the circadian rhythm moved forward, advancing the sleeping and waking times of zebrafish, possibly because alterations in melatonin secretion caused inflammation and sleep disorders.

Our results support that *K. pneumoniae* infection disrupts the circadian rhythm through inflammation (Fig. 7). The inflammatory response to infection is mediated by the release of pro-inflammatory cytokines, which are important signaling molecules in the fight against and prevention of infection transmission [42,43]. In this study, *K. pneumoniae* infection increased the recruitment of neutrophils and macrophages that produce pro-inflammatory factors and the expression of the related genes *il1b*, *nfkb*, *tnfa*, and *tgfb1a* in zebrafish under L/D conditions. The anti-inflammatory drug G6PDi-1 rescued the expression of clock genes by reducing the production of inflammatory cytokines (Fig. 7). *Klebsiella pneumoniae* infection increases the levels of inflammatory cytokines and immune cells, which help to clear the pathogenic bacteria after infection. Activation and inhibition of the pro-inflammatory factor NF-κB increases and decreases the circadian amplitude of *per3*, respectively [44]. TNF-α inhibits the expression of clock genes by interfering with E-box-mediated transcription [45]. Therefore, *K. pneumoniae* may directly affect circadian rhythms by activating inflammatory signaling pathways and acting on clock genes. The circadian rhythm directly regulates the expression of several inflammatory factors. We found that the expression of *il1b*, *nfkb*, and *tnfa* had an obvious rhythmic pattern, which is consistent with the results of previous studies. Melatonin also reduces inflammation and apoptosis after infection [46]. Therefore, after infection with *K. pneumoniae*, melatonin secretion may increase through circadian rhythm regulation for self-protection. In addition, melatonin restricts leukocyte recruitment, which may explain the suppression of the innate immune response under D/D conditions [47,48]. The SCN is the main pacemaker of the circadian rhythm, and inflammatory mediators can act on the SCN [49,50]. Although zebrafish do not possess a SCN, they have similar functional structures [27]. However, the *in vivo* interactions between bacterial products acting directly on the SCN and indirectly on the inflammatory mediators released by the immune system remain unclear. Clinical reports have shown that *K. pneumoniae* can invade the brain and cause significant brain abscesses [51]. Whether *K. pneumoniae* can directly affect the SCN and cause disturbances in the circadian rhythm requires further study.

Klebsiella pneumoniae regulates the circadian rhythm via inflammation, in which light plays an important role (Fig. 7). Light can help reset and synchronize the circadian rhythm as well as enhance the production of pro-inflammatory cytokines and recruitment of innate immune cells to an infection site [52,53]. The increased survival of zebrafish larvae infected with *Salmonella enterica* in the presence of light is associated with increased bacterial clearance, expression of inflammatory cytokines, and recruitment of neutrophils and macrophages to infection sites [52]. In this study, the number of neutrophils and macrophages in zebrafish treated with *K. pneumoniae* was increased under L/D conditions, and the expression of inflammation-related genes was upregulated; however, under D/D conditions, immune cell numbers were nearly unchanged compared with those in the control group, and the expression of inflammation-related genes was downregulated. Therefore, compared with the D/D condition, the L/D condition was more likely to disturb the circadian rhythm, which may be related to greater induction inflammatory cells and inflammatory factors in the presence of light. Previous studies have indicated that light regulation plays an important role in reducing inflammation in patients; further, infrared light therapy has recently been used to relieve TLR-4-dependent hyperinflammation induced by COVID-19 [54,55]. Therefore, in the clinical treatment of inflammation, light factors may be useful as an auxiliary therapy. Additionally, the effect of light on immune function may be related to the rhythmic expression of innate immune receptors involved in recognizing gram-negative bacteria. For example, phagocytosis of *Escherichia coli* by zebrafish myeloid cells peaks in the light stage and decreases to a trough at night,

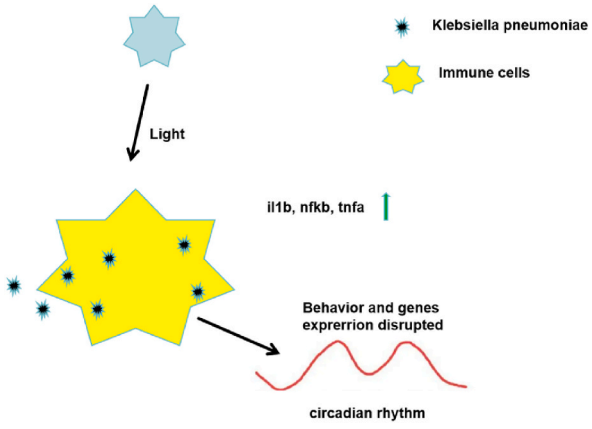

Fig. 7. Proposed scheme of how *Klebsiella pneumoniae* alters zebrafish circadian rhythm via inflammatory pathways dependent on light cues.

whereas phagocytosis of *Staphylococcus aureus* shows no noticeable shock [56]. The NTUH-K2044 strain is a Gram-negative strain of *K. pneumoniae*. The circadian rhythm can be regulated by light, and many circadian rhythm genes can be directly affected by light. Normal light can maintain more stable rhythmic expression and rhythmic signal output of the circadian rhythm, thereby maintaining homeostasis of various physiological reactions in the body.

This study has some limitations. First, we used zebrafish as a model organism to investigate the impact of *K. pneumoniae* infection on biological rhythms. Although zebrafish are important in biomedical research, their physiological differences with mammals may limit the clinical applicability of our results. Second, although zebrafish is a very useful model organism, the lack of suitable antibodies compared to those in humans and rodents limits research in zebrafish. Third, we found that light plays an important role in disrupting the biological rhythm of inflammation after *K. pneumoniae* infection; however, the underlying detailed mechanism requires further investigation.

Nonetheless, the impact of *K. pneumoniae* infection on the circadian clock in clinical practice has not been previously reported. We found that *Klebsiella pneumoniae* infection leads to substantial changes in other physiological processes, such as blood pressure, respiration, and sleep. The circadian clock is an internal rhythmic time device, and its disruption can indirectly affect other biological processes. The inflammatory response after *K. pneumoniae* infection occurs as an initial response and plays a crucial role in clearing bacteria. Light increases the generation of inflammatory factors and inflammatory cells. Whether increased light can be used to treat *K. pneumoniae* infection should be further examined.

5. Conclusion

Here, our findings revealed that *Klebsiella pneumoniae* disrupts the circadian rhythm in zebrafish. During this process, light positively regulates the immune response of juvenile zebrafish to bacterial infection and enhances the recruitment of neutrophils and macrophages as well as the production of inflammatory factors. An anti-inflammatory drug treatment restored inflammation levels and rescued the disruption of circadian rhythm-related gene expression in zebrafish. These results show that *K. pneumoniae* affects the zebrafish circadian rhythm through inflammation and that light plays an important role in this process. Our results revealing a relationship between *K. pneumoniae*, inflammation, and the circadian rhythm provide a theoretical reference for studying circadian rhythm disorders caused by inflammation.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Hui Ding: Validation, Methodology, Investigation, Data curation. **Xiao-chun Chen:** Software, Resources, Methodology, Formal analysis, Data curation. **Lin Wan:** Writing – original draft, Resources, Methodology, Investigation, Data curation. **Ying-ying Zhang:** Resources, Investigation, Data curation. **Xiao-hong Rui:** Software, Resources, Methodology, Data curation. **Tian He:** Formal analysis, Data curation, Conceptualization. **Jun Liu:** Writing – review & editing, Writing – original draft, Validation, Supervision, Data curation, Conceptualization. **Zhong-bo Shang:** Writing – original draft, Supervision, Methodology, Investigation, Data curation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Jun Liu reports financial support was provided by Affiliated Wuxi Fifth Hospital of Jiangnan University that includes: employment. No If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30829.

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