Original Article

Effect of circadian rhythm disruption induced by time-restricted feeding and exercise on oxidative stress and immune in mice

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Frequent or long-term circadian disorders can lead to a range of health problems, including chronic insomnia, depression, chronic diseases, and cancer. It has also been shown that altering the feeding time of mice from night to day can result in circadian disorder. Recent studies have revealed complex interactions between circadian rhythm and oxidative stress. However, little is known about the impact of circadian rhythm disorders caused by time-restricted feeding on mental state, immune function, and oxidative DNA damage. In this study, we investigated the effects of circadian rhythm disruption by controlling the timing of feeding and exercise on oxidative DNA damage and immune responses in 8-week-old mice for 14 days. Body weight, daytime running wheel activity, serum interleukin-6 levels, urinary 8-hydroxy-2'-deoxyguanosine levels, and nuclear DNA (liver, lung, testes, and pancreas) were significantly increased in the night-restricted group compared with the control group. Additionally, the mice in the night-restricted group exhibited anxiety-like behavior. These results indicated that the circadian rhythm disruption due to abnormal dietary timing can lead to obesity, mental state dysregulation, immune function changes and oxidative DNA damage in mice. This oxidative DNA damage may contribute to the initiation and increased risk of cancer.

Key Words: **circadian rhythm, oxidative DNA damage, running wheel activity, 8-hydroxy-2'-deoxyguanosine, interleukin-6**

Circadian rhythms are biological cycles with a period of approximately 24-h that regulate daily oscillations in behavior and critical physiological processes in mammals. These rhythms play a vital role in maintaining physiological homeostasis and normal functioning in humans, influencing mental and physical states, behavior, and mood. Dysregulation of the circadian rhythm has been linked to various health problems, including an increased risk of psychiatric, neurological, cardiometabolic, and immune system disorders, as well as reproductive issues and mood disorders.(1) Notably, the World Health Organization has classified circadian rhythmicity dysregulation as a probable carcinogen based on evidence from both population and laboratory studies. Understanding the mechanisms by which disrupted circadian rhythms promote tumor development is an area of active research.

Recently, circadian rhythms have emerged as a critical factor in the field of nutrition. Growing evidence suggests that the quality, timing, and quantity of food intake significantly affect the circadian system. Conversely, circadian rhythms also influence nutrient metabolism.(2,3) The interaction between circadian rhythm and nutrition can influence metabolic disorders, obesity, and exercise performance optimization. Food intake time is considered a potential synchronizer that entrains metabolic peripheral clocks within the body, aligning them with the central block in the brain.(4) Recent studies have shown that altering the feeding time of mice from night to day disrupts this synchrony, with a mismatch in the expression of circadian rhythm genes between the two clocks, leading to circadian rhythm disruption.(5,6) Understanding this intricate relationship is vital for improving overall health and developing effective dietary strategies.

Oxidative stress arises from an imbalance between the production of reactive oxygen species (ROS) produced in cells and tissues and the capacity of the body's antioxidant defenses to neutralize them. ROS are primarily generated as byproducts of the mitochondrial electron transport chain and act as signaling molecules to regulate physiological and biological processes.(7) However, ROS can also be produced in response to various stressors, including mental stress, inflammation, infection, and exposure to toxins.(8–10) When not adequately neutralized by antioxidants, excessive ROS can damage proteins, lipids, and nucleic acids, contributing to the development of metabolic disorders, neurological conditions, and cancer.(11,12) 8-hydroxy-2'-deoxyguanosine (8-OHdG) is a major product of oxidative DNA damage and a well-established biomarker for oxidative stress and DNA damage.(13) It is commonly measured in blood, urine, and tissues to assess oxidative stress and serves as a potential indicator for diseases such as cancer, cardiovascular diseases, and neurodegenerative disorders.(14–16) Recent research has revealed a complex interplay between circadian rhythms and oxidative stress. Circadian rhythms can influence ROS production and detoxification, while oxidative stress can disrupt these rhythms at the molecular level. Understanding this connection is essential for developing effective strategies to prevent or treat diseases associated with circadian rhythm disruption and oxidative stress.

Interleukin-6 (IL-6) is a pleiotropic cytokine produced by various immune and non-immune cells. It plays a crucial role in both innate and adaptive immune responses. Produced in response to infection and injury, IL-6 contributes to both acute and chronic inflammation. Elevated levels of IL-6 have been linked to several chronic diseases, including cardiovascular diseases, diabetes, and cancer. Vgontzas *et al.*(17) demonstrated that sleep loss was correlated with an increase in IL-6 levels the following day. IL-6 is also associate with stress-related disorders such as depression and anxiety.(18,19)

The mechanisms by which circadian rhythm dysregulation impacts health, particularly its relationship with oxidative stress and the immune system, have not been fully elucidated. This study employs a mouse model of circadian rhythm dysregulation

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Table 1. Characteristics of the animals

Characteristics	Group	Administration period (day)		
		0	7	14
Body weight (24 h)	C	1	0.98 ± 0.02	1.01 ± 0.03
	CD	1	0.99 ± 0.02	1.02 ± 0.06
	ND	1	1.04 ± 0.03**,‡	1.07 ± 0.04*,†
Water consumption (24 h)	C	1	1.36 ± 0.34	1.48 ± 0.55
	CD	1	1.60 ± 0.33	1.24 ± 0.36
	ND	1	1.12 ± 0.12	1.00 ± 0.40
Diet intake (24 h)	C	1	1.09 ± 0.31	0.93 ± 0.30
	CD	1	1.11 ± 0.30	0.78 ± 0.38
	ND	1	0.97 ± 0.18	0.76 ± 0.24
Urine (24 h)	C	1	1.53 ± 0.62	1.58 ± 1.25
	CD	1	2.11 ± 0.86	1.82 ± 0.77
	ND	1	2.50 ± 1.52	1.38 ± 1.09
Feces (24 h)	C	1	0.89 ± 0.25	0.88 ± 0.21
	CD	1	0.70 ± 0.28	0.74 ± 0.10
	ND	1	0.91 ± 0.38	0.76 ± 0.19

C, unrestricted control group; CD, day-restricted diet and exercise group; ND, night-restricted diet and exercise group. Day 0 values were measured just before the initiation start of the experiments. Day 7 and 14 values are expressed as ratios to the day 0 values. Compared to the C group: *p<0.05, **p<0.01. Compared to the CD group: †p<0.05, ‡p<0.01.

Table 2. Tissue weight of the animals on day 14 after dietary and exercise restriction

Group	Tissue				
	Liver/BW (%)	Kidney/BW (%)	Spleen/BW (%)	Lung/BW (%)	Testes/BW (%)
C	5.06 ± 0.33	1.68 ± 0.09	0.40 ± 0.04	0.65 ± 0.08	0.72 ± 0.12
CD	6.16 ± 0.45**	1.74 ± 0.12	0.42 ± 0.04	0.66 ± 0.02	0.74 ± 0.05
ND	5.21 ± 0.43‡	1.77 ± 0.11	0.37 ± 0.02†	0.62 ± 0.02†	0.77 ± 0.08

BW, body weight; C, exercise restriction in unrestricted control group; CD, day-restricted diet and exercise group; ND, night-restricted diet and exercise group. Compared to the C group: *p<0.05, **p<0.01. Compared to the CD group: †p<0.05, ‡p<0.01.

induced by time-restricted feeding and spontaneous exercise. We aimed to utilize this model to evaluate the effects of circadian rhythm disruption on oxidative DNA damage and the immune system.

Materials and Methods

Animal experiments. Eight-week-old male BALB/c mice were obtained from SLC Japan Inc. (Shizuoka, Japan). BALB/c mice are a commonly used inbred strain in studies pertaining to cancer, infectious disease, immunity, and mental disorders. Fifteen mice were divided into three groups (n = 5 per group): the control group (C group) had unrestricted access to both a standard diet and running wheel throughout the day (24-h access); the day-restricted group (CD group) was allowed access to the running wheel and a standard diet only during the nighttime (12-h access, 7:00 p.m. to 7:00 a.m., with limited access from 7:00 a.m. to 7:00 p.m.); and the night-restricted group (ND group) was allowed access to the running wheel and a standard diet only during the daytime (12-h access, 7:00 a.m. to 7:00 p.m., with limited access from 7:00 p.m. to 7:00 a.m.). Running wheel activity was measured using custom-made cages (Bio Research Center Co., Ltd., Nagoya, Japan) equipped with running wheels. The running wheel activity was continuously recorded every 4 s using the LabVIEW 13.0 software (National Instruments, Tokyo, Japan) for 14 days. The mice were individually housed in metabolic cages for 24 h to collect urine at 0, 7, and 14 days after the initiation of the diet and running wheel protocol. At the time of urine collection, their daily body weight, food intake, water consumption, fecal output and urine output were also measured. After 14 days of protocol, the mice were euthanized between 1:00 p.m. and 3:00 p.m. Serum and organs of interest were collected and stored at −80°C for further analysis. Urine samples were stored at −20°C until measurement of 8-OHdG levels.

Throughout the experiment, the mice were fed a commercially available diet free of 8-OHdG (Dyet no. 110952; Dyets Inc., Bethlehem, PA) and tap water. The mice were housed in a temperature-controlled room (25°C) with a 12-h light/dark cycle for the duration of the experiment. The light cycle was 12 h, with lights on from 7:00 a.m. to 7:00 p.m. and lights off from 7:00 p.m. to 7:00 a.m. All animal experimental procedures were performed according to the guidelines established in the Japanese Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee, University of Occupational and Environmental Health, Japan (AE 21-009; June 11, 2021).

Open-field test. Anxiety-like behavior was evaluated using an open-field test on days 0, 7, and 14, following the initiation of the feeding and running wheel protocol. The test was conducted between 1:30 p.m. and 5:00 p.m. to minimize circadian rhythm influences. The open-field apparatus was a square chamber measuring 40 cm × 40 cm × 32 cm. A central area measuring 20 cm × 20 cm was marked on the arena floor. Three hours before the test, the mice were transferred to the testing room for habituation. Each mouse was gently placed in a corner of the chamber, and their behavior was video-recorded for 15 min using

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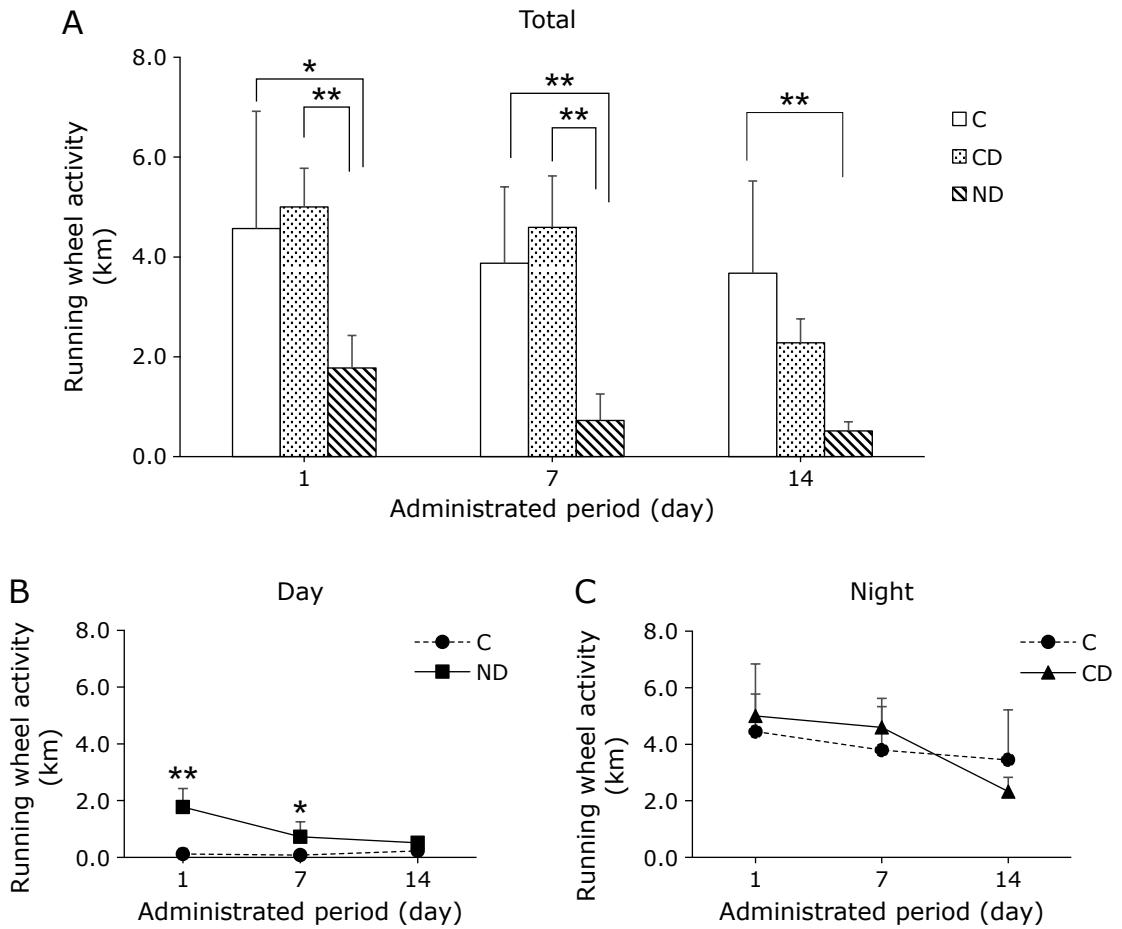

Fig. 1. Effect of circadian rhythm disruption caused by dietary restriction on running wheel activity. A: Total running wheel activity of BALB/C mice on day 1, 7, 14 after dietary and exercise restriction for unrestricted control (C), day-restricted (CD), and night-restricted (ND) groups. B: Running wheel activity from 7:00 a.m. to 7:00 p.m. C: Running wheel activity from 7:00 p.m. to 7:00 a.m. Values are represented as the mean ± SD (n = 5). *p<0.05 and **p<0.01 using one-way analysis of variance (ANOVA).

the Ethovision® XT 15.0 software (BrainScience idea Co., Ltd., Osaka, Japan). The total distance moved, the number of entries into the central area, and the time spent in the center were monitored and recorded. The chamber was cleaned with 70% ethanol after each test.

Analysis of serum IL-6. Serum IL-6 levels were measured using a mouse IL-6 enzyme-linked immunosorbent assay kit (Proteintech Group Inc., Rosemont, IL) according to the manufacturer's instructions.

Analysis of urinary 8-OHdG. Urinary 8-OHdG levels were analyzed using a previously described high-performance liquid chromatography system with electrochemical detection.[20,21] Urinary creatinine levels were used to normalize 8-OHdG concentrations.

Analysis of 8-OHdG in tissue DNA. Nuclear DNA was isolated from 100 mg of tissue using a DNA Extraction WB Kit (FUJIFILM Wako Pure Chemical Co., Ltd., Osaka, Japan). 8-OHdG levels in the tissue DNA were determined using our previously described method.[10] The results are expressed as the number of 8-OHdG molecules per 10^6 deoxyguanosine molecules.

Statistical analysis. All data were analyzed using one-way analysis of variance to assess individual differences with the IBM SPSS 29 statistics software package (SPSS Inc., Chicago, IL). Data are presented as the mean ± SD. Statistical significance was set at *p<0.05 and **p<0.01.

Results

Body weight, water consumption, food intake, urine output, and tissue weight. Mice in the ND group showed a significant increase in body weight compared to the C group on days 7 and 14 (p = 0.001, p = 0.043) (Table 1). No significant differences were observed in water consumption, food intake, urinary output, or fecal output between the three groups. Interestingly, the weights of the liver, spleen, and lungs were significantly lower in the ND group mice compared to the CD group on day 14 (Table 2). The CD group also had significantly higher liver weights compared to the C group.

Running wheel activity. As expected for nocturnal animals, there was no significant difference in running wheel activity between the C group over the whole day (24-h) or night (12-h) and the CD group during the night (12-h) (Fig. 1A and C). However, the ND group exhibited significantly higher daytime (12-h) running wheel activity compared to the C group throughout the experiment (Fig. 1B). Overall, the running wheel activities of the C group over the entire day (24-h) and the CD group during the night (12-h) were significantly higher than those of the ND group during the daytime (12-h) (Fig. 1A). Running wheel activity decreased over time in all three groups, with a more rapid decline observed in the ND group (59% and 71% reduction on days 7 and 14, respectively, compared to day 1).

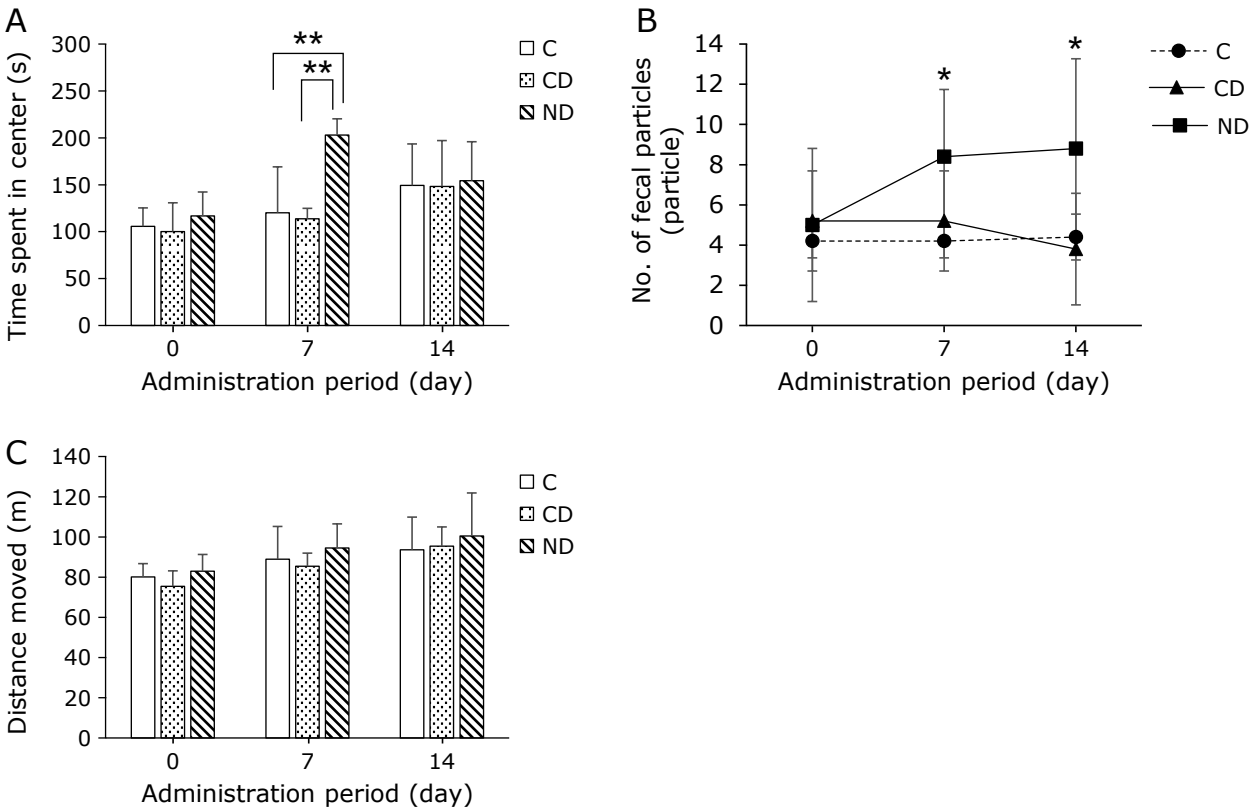

Fig. 2. Behavior in open-field test. A: Time spent in the center of the chamber. B: Number of fecal particles. C: The total distance moved. Values are represented as the mean ± SD (n = 5). **p<0.01 (ANOVA).

Anxiety-like behavior. Open-field testing was used to assess spontaneous activities and anxiety-like behavior. While the ND group spent significantly more time in the central area of the chamber and produced a greater number of fecal pellets 7 days after the experiment began (Fig. 2A and B), no significant difference was observed in the total distance moved between the three groups (Fig. 2C). Specifically, the time spent in the center area increased by 69% in the ND group compared to the C group. These findings suggest a trend towards increased anxiety-like behavior in the ND group, although the time spent in the center area did not differ between the three groups at day 14.

Serum IL-6 levels. IL-6 levels were significantly higher in the ND group compared to the C and CD groups at day 14 days (Fig. 3). IL-6 levels in the ND group were 1.34 times higher than those in the C group. No significant differences were observed between the C and CD groups.

8-OHdG levels. As an indicator of oxidative DNA damage, 8-OHdG levels in the urine and tissue DNA were measured. Urinary 8-OHdG levels in the ND group significantly increased throughout the experiment (Fig. 4A). The levels in the ND group were 1.24 and 1.21 times higher than those in the C group on days 7 and 14, respectively. Nuclear DNA 8-OHdG levels in the liver, lungs, testes, and pancreas of the ND group were also significantly increased 14 days after dietary restriction during the dark period (Fig. 4B). The 8-OHdG levels in these tissues of the ND group were 1.22 to 1.50 times higher than those in the C group. However, nuclear DNA 8-OHdG levels in the brain, kidney, and spleen of the ND group did not differ significantly from those in the C or CD groups (data not shown).

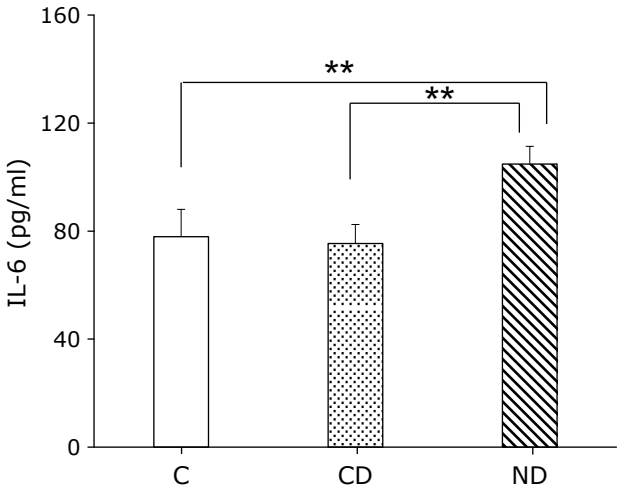

Fig. 3. Effect of circadian rhythm disruption on serum interleukin-6 levels on day 14 after dietary restriction in the three groups (C, CD, and ND). Values are represented as the mean ± SD (n = 5). **p<0.01 (ANOVA).

Discussion

Research has shown that restricting nocturnal food intake in mice can lead to disturbances in circadian rhythms.[6] In our study, we investigated the effects of disrupting circadian rhythms in mice by restricting their food intake and exercise activity to the daytime. We assessed oxidative stress by measuring 8-OHdG

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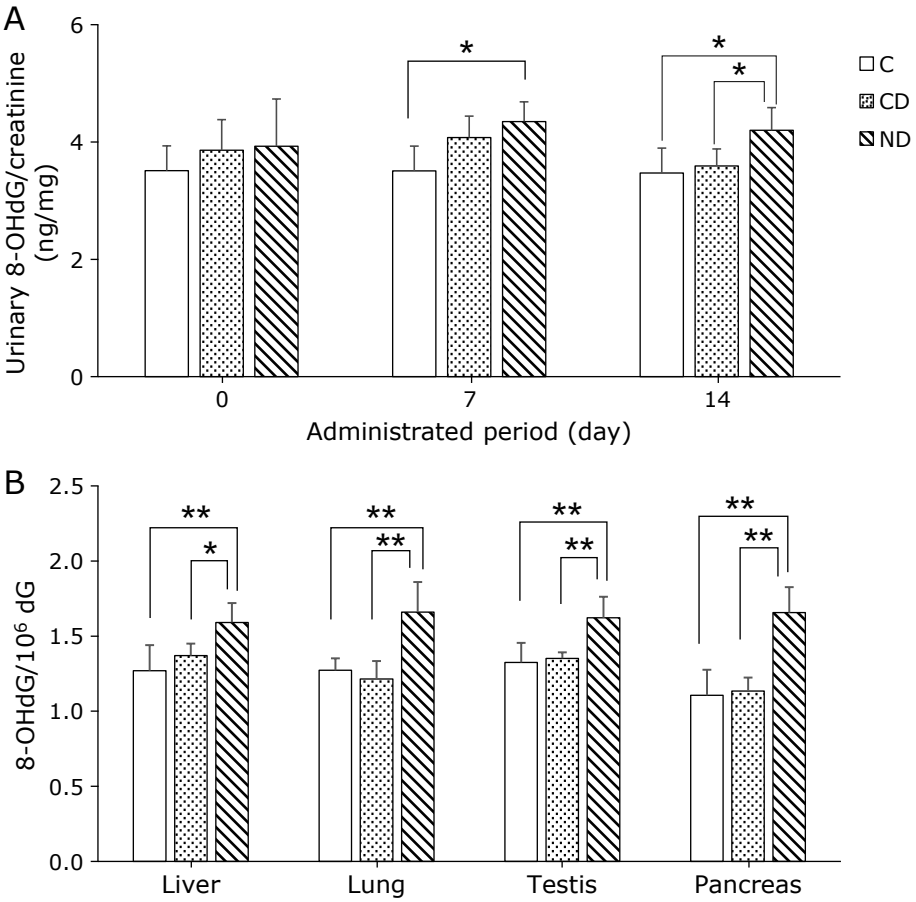

Fig. 4. Effect of circadian rhythm disruption caused by dietary restriction on 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in the three groups (C, CD, and ND). A: Urinary 8-OHdG levels. B: 8-OHdG levels in tissue DNA on day 14 after dietary restriction. Tissue DNA values were calculated as the number of 8-OHdG molecules per 10^6 deoxyguanosine (dG) molecules. Values are represented as the mean ± SD ($n = 5$). *$p<0.05$ and **$p<0.01$ (ANOVA).

levels in urine and tissue DNA. Additionally, we evaluated the influence of the circadian rhythms disruption on stress and immunity using open-field testing and measuring IL-6 levels, respectively.

We observed a significant increase in body weight when the mice changed their feeding time from night to day. However, no significant differences in daily food intake were observed among the three groups. This aligns with another study that reported weight gain in mice fed a high-fat diet only during the day compared to those fed the same diet at night.[22] The caloric intake was not significantly different between the two groups. However, mice fed during the day moved less than those fed at night. We observed similar results to this study, where the running wheel activities in the ND group during the day were significantly lower than those in the C and CD groups during the night. Weight gain in the light-fed group may be attributed to a combination of similar calorie intake and a significant reduction in running wheel activity. Turek *et al.*[23] found that mice that are homozygous mutants of clock genes exhibit prolonged circadian rhythms and persistent loss of rhythm and have significantly higher body weights despite similar daily food intake compared to wild-type mice. Therefore, circadian rhythm disruption may be a risk factor for weight gain and obesity, similar to an unhealthy diet. Studies have also indicated that circadian rhythm dysfunction can lead to decreased levels of leptin, the satiety hormone, or leptin resistance, potentially leading to obesity.[24,25] However, Greenwell *et al.*[26] suggested that the body weight of C57BL/6J male mice subjected to restricted feeding at night was not significantly different from that of unrestricted. This variation may be attributed to differences in the mouse strains.

Qian *et al.*[27] found that workers who simulated nighttime work combined with both daytime and nighttime eating patterns experienced a 26.2% increase in depression-like symptoms and a 16.1% increase in anxiety-like symptoms compared to daytime workers. However, this increase was not observed in the workers who simulated nighttime work with daytime-only eating patterns. Timing of food intake based on a regular schedule synchronized with circadian rhythms can minimize mood fluctuations in individuals experiencing circadian rhythm disruptions. We observed similar results in this study. The time spent in the central area of the open field by the ND group was significantly increased on day 7 in the open-field test. However, there was no significant difference in the total distance moved compared with the C group. Studies have shown that an increase in central movement or time spent in the central part of the open field chamber, without changes in total movement or vertical exploration can indicate anxiety-like behaviors.[28,29] Although there was no significant difference in the daily fecal output between the ND and the control group, the mice in the ND group excreted more fecal pellets during the open-field test. Fecal excretion is also considered an anxiety-like behavior indicator in open-field testing. These results indicate that night-feeding-restricted mice display significant anxiety-like behaviors. Monje *et al.*[18] showed that mice subjected to long-term light deprivation with constant darkness for 4 weeks displayed depression-like behavior and elevated levels of plasma and hippocampal IL-6. Additionally, the authors

observed altered hippocampal protein levels of the clock-related genes *Per2* and *Npas2*. Mice lacking IL-6 showed resistance to depression-like behavior induced by long-term light deprivation, indicating a crucial role for IL-6 in the development of depression induced by constant darkness. Adams *et al.*[30] reported that IL-6 levels in the blood of mice fluctuate throughout the day and are significantly higher during the light period than during the dark period, peaking at 10:00 a.m. A 6-h phase advance of the light-dark cycle, once a week for 4 weeks, significantly elevated IL-6 levels. Our study also demonstrated that IL-6 levels in the serum collected between 1:00 p.m. and 3:00 p.m. were significantly elevated in the ND group. This may be linked to anxiety-like behaviors and circadian rhythm disruptions caused by dietary restriction.

Increasing evidence has shown that the circadian clock maintains a balance between the antioxidant system and ROS production. Disruptions in the circadian rhythm can lead to oxidative stress and disturbances in the redox balance.[31,32] Accumulation of oxidative stress is observed in clock disruption models and shift workers and is considered a risk factor for various diseases linked to circadian rhythm disruption. A recent study found that changes in the timing of food intake or composition of food in mice could significantly affect their circadian rhythm. Daytime feeding reversed circadian gene expression in the peripheral clock in the liver but did not change expression in the central clock.[33] This indicates that daytime feeding can induce circadian disorders in mice. In this study, we obtained similar results with elevated urinary 8-OHdG levels in the ND group due to dietary restriction at night. Some studies have reported that urinary levels of 8-OHdG in nightshift workers were significantly higher than those in day shift workers.[34,35] In this study, 8-OHdG levels in the nuclear DNA in the liver, lung, testes, and pancreas were also significantly increased in the ND group. Epidemiological studies have indicated that circadian rhythm disruptions are associated with an increased cancer risk, including cancers of the liver, pancreas, and lung.[36–38] Demirkol *et al.*[39] indicated that semen quality in men who work shifts can be impaired without a corresponding decrease in testosterone levels. The health effects of circadian rhythm disruption may be linked to the accumulation of oxidative DNA damage in the affected organs. Circadian regulation of glutathione and oxidative stress-related enzymes, including glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase, has been confirmed.[40–42] Disruption of the circadian rhythm may also interfere with the activity patterns of antioxidant enzymes, leading to the accumulation of oxidative DNA damage. However, this association requires further investigation.

In this study, we used a mouse model to investigate the effects of circadian rhythm disruption on oxidative stress and immunity. Mice with day-time food intake and exercise activity showed a significant increase in body weight, IL-6 levels, and oxidative DNA damage due to circadian rhythm disruption. The accumulation of oxidative DNA damage and IL-6 may contribute to the health risks associated with circadian rhythm disorders. However, further Studies are required to understand the precise mechanisms by which the circadian clock affects oxidative DNA damage and immunity. We also need to assess more immunity markers and behavioral changes. A better understanding of this relationship can provide a theoretical basis for establishing more scientific and reasonable lifestyles and work patterns, thereby reducing health risks for night shift workers. Although circadian rhythms are evolutionarily conserved in mice, these findings still need to be validated in animals with sleep patterns similar to those of humans. This study offers a feasible animal model for investigating the impact of circadian rhythm disorders on health, paving the way for future research in this important area.

Author Contributions

Y-SL, HF, KF, and KK designed and critically discussed the study. Animal experiment, Y-SL and HF; Project administration and data analysis, Y-SL and HF; Methodology, Y-SL and HF; Supervision, KF; Validation, KK and KF; Writing, Y-SL; Obtained funding, Y-SL. All authors have read and approved the final manuscript.

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Abbreviations

IL-6	interleukin-6
8-OHdG	8-hydroxy-2'-deoxyguanosine
ROS	reactive oxygen species

Conflict of Interest

No potential conflicts of interest were disclosed.

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