

Letter

## Combined effect of circadian dysfunction and cadmium on immune suppression

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**ABSTRACT** — Most living beings including human beings have endogenous 24-hr rhythms, called circadian rhythms. The most important factor to alter the circadian rhythms is light. Therefore, this biological rhythms can easily disrupt by exposure to light at night (LAN). In modern society, rotating shift work with night work (night shift work) is essential working arrangement and induces circadian disruption to workers by LAN exposure. Epidemiologic studies indicate that a long-term biological rhythm disruption induced by a long-term LAN exposure causes many serious health disorders including immune dysfunction. Cd also dysregulates the immune system. Main exposure source of Cd is tobacco smoking and food intake in living environment. Further, in work places with handling Cd, occupational environment also be an exposure source with high concentration of Cd. In this report, we paid attention to and examined the combined effect of Cd and LAN on immune system. Mice were kept under the light/dark shift condition and/or injected CdCl<sub>2</sub> (0.33 or 1.0 mg/kg) twice a week for 4 weeks followed by sacrificing 7 days after the last injection. The mitogen activity induced by phytohemagglutinin was markedly reduced by shift condition; further, this reduced mitogen activity was completely inhibited by additional Cd treatment. This result indicates a possibility of enhancement of immune dysfunction by Cd and LAN combination. Our result suggests that Cd exposure when being the circadian disruption had potential for more inhibition of immune function.

**Key words:** Chronotoxicity, Cadmium chloride, Rotating shift work, Mitogen activity, Immune toxicity, Circadian rhythm

### INTRODUCTION

Many organisms including human beings have endogenous 24-hr rhythms, called circadian rhythms, which are endogenous 24-hr variations found in various physiological processes. This biological rhythms are synchronized to light/dark cycle, therefore the most important factor to alter the circadian rhythms is light. Especially, exposure to light at night (LAN) easily leads to disrupt this biological

rhythms (Foster and Kreitzman, 2014).

Rotating shift work with night work (night shift work) has become an indispensable working arrangement of today's 24-hr society. The night shift work may contribute to disrupt the biological rhythms of workers who engage in the night shift work, because workers receive LAN exposure. Epidemiologic studies have been reported that the night shift work is a major risk factor for many serious health disorders, such as obesity (Karlsson *et al.*,

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2001), type II diabetes (Morikawa *et al.*, 2005), hypertension (Esquirol *et al.*, 2011), gastric ulcers (Knutsson and Boggild, 2010; Segawa *et al.*, 1987), as well as several types of cancer including breast (Davis *et al.*, 2001; Schernhammer *et al.*, 2001; Stevens *et al.*, 2013), prostate (Conlon *et al.*, 2007; Kubo *et al.*, 2011; Kubo *et al.*, 2006), and colon (Kloog *et al.*, 2009; Schernhammer *et al.*, 2003).

Dysregulation of the immune system is one of the common feature of biological dysfunction induced by circadian disruption (Castanon-Cervantes *et al.*, 2010). Indeed, chronic circadian disruption alters sleep/wake cycle, thereby abnormally changed several immunological parameters including serum cytokine levels, lymphocytes, and Ig classes in animal models and humans (Everson, 2005; Hui *et al.*, 2007). It is well known that exposure to Cd alters immune system, e.g., immunosuppressive effects (Blakley, 1985) and IgE synthesis inhibition (Marth *et al.*, 2001), suggesting that the immune systems is vulnerable to Cd toxicity (Blakley, 1985). Humans can be exposed to Cd through smoking tobacco and by consuming contaminated foodstuffs, such as rice and wheat harvested from soil polluted with Cd (Miura, 2009). Interestingly, a combined exposure of Cd from both occupational and living environment provoked a higher prevalence of renal dysfunction, the well-known Cd-induced health damage (Jin *et al.*, 2004). Therefore we paid attention to the combined effect of Cd and LAN on immune system. In this report, we prepared circadian disruption using shift work model with or without Cd injection, thereafter, estimated the combined effect of these stresses on immune function.

## MATERIALS AND METHODS

### Chemicals

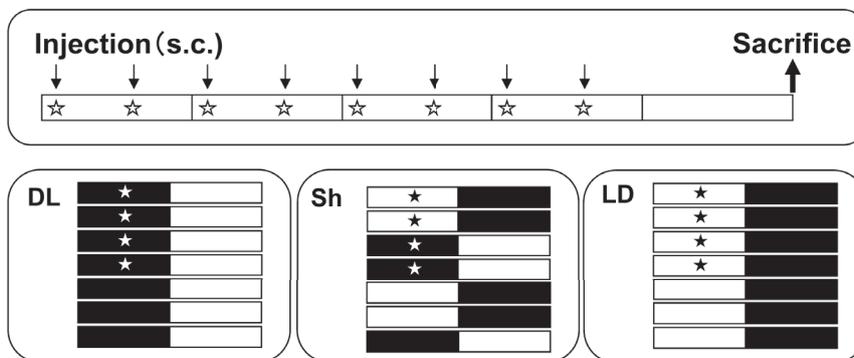
Cadmium chloride was purchased from Wako Pure

Chemical (Osaka, Japan). Fetal bovine serum (FBS) was purchased from ICN Biomedicals Inc. (Aurora, OH, USA). RPMI medium was purchased from Invitrogen (Grand Island, USA). Sterile saline was purchased from Otsuka Pharmaceutical (Tokyo, Japan). Water soluble tetrazolium salt (WST-5: 2,2'-Dibenzothiazolyl-5,5'-bis[4-di(2-sulfoethyl)carbamoylphenyl]-3,3'-(3,3'-dimethoxy 4,4'-biphenylene) ditetrazolium, disodium salt) was purchased from Dojindo (Kumamoto, Japan). Phytohemagglutinin (PHA-M) was purchased from Cell Biology Boehringer Mannheim GmbH (Mannheim, Germany).

### Animals, lighting conditions, and Cd injection

Male C57BL/6J mice (5 weeks of age) were purchased from Nihon Clea (Tokyo, Japan), and were kept under standard condition with controlled temperature ( $24 \pm 1^\circ\text{C}$ ), humidity ( $55 \pm 5\%$ ) and light (12:12 hr light/dark cycles). The lights were white fluorescent lamp, about 100-150 lux intensity at the level of cages. All animals had free access to sterilized commercial pellet diet (CE-2, Clea Japan, Inc., Tokyo, Japan) and sterilized filtered tap water. The three breeding rooms were prepared to explore the effect of light/dark condition on immune system: light-dark condition (LD), lights on at 08:00 AM; dark-light condition (DL), lights on at 08:00 PM; and shift condition (Sh), lights on at 08:00 AM for 2 days and conversely following 2 days, lights on at 08:00 PM (Fig. 1). Before experiments, mice were kept for 14 days under the each condition to adapt them to their lightning conditions.

To examine the cadmium (Cd)-induced immune toxicity, mice (7 weeks of age,  $n = 5$ ) kept under the each condition were received subcutaneous (s.c.) injection of 0.33 or 1.0 mg/kg of  $\text{CdCl}_2$  (1.7 or 5.2  $\mu\text{mol Cd/kg}$ , respectively) dissolved in saline twice a week for 4 weeks. Control mice were received an equal volume (0.1 mL/10 g body



**Fig. 1.** A schema of experimental design including lightning conditions and injection schedule.

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weight) of saline. Each injection time was: ZT6 (ZT: zeitgeber time, indicated for elapsed time from the lighting on) for LD condition; ZT18 for DL condition; ZT6 and ZT18 for Sh condition (Fig. 1). Mice were sacrificed 7 days after the last injection. Spleen organs were separated and weighed immediately.

These animal experiments were conducted under the guidelines for the care and use of laboratory animals set forth by our Institutional Animal Care and Use Committee of the National Institute of Occupational Safety and Health, Japan (JNIOHS).

### Mitogen activity

Spleen was immediately removed after the sacrifice, and was minced with scissors to release spleen cells in 2 mL of RPMI medium containing 2 mM glutamine and 5% FBS. Spleen cells ( $1 \times 10^6$  cells/mL, 100  $\mu$ L/well) were cultured in 96-well microplate with 4  $\mu$ g/mL of PHA-M for 4 days to activate T cells. In this assay, PHA-M used as mitogen induced T cell activation (Wietzerbin *et al.*, 1978). Water soluble tetrazolium (WST-5, 0.3 mg/mL, final concentration) was added and incubated for 4 hr followed by measuring the absorbance at 450 nm by microplate reader (Immune Reader NJ-2100, Inter Med, Tokyo, Japan).

### Statistical analysis

Data were analyzed by one-way ANOVA. Statistical significance of difference between all the control and Cd-treated groups in the three light/dark cycle conditions was determined with Fisher's PLSD test. In all cases,  $p < 0.05$  was considered statistically significant.

## RESULTS

We confirmed that the Sh condition used in this experiment clearly disrupted circadian rhythms of mice, using infrared temperature sensor which estimated the behavioral activity (data not shown). Figure 2 shows the body weights measured 7 days after the last injection. In the saline treatment group (Control), the body weights showed no significant differences among LD, DL, and Sh conditions, indicating that the light/dark condition did not give any impact on body weight. The body weights in the Cd treatment group basically did not significantly changed, although it was slightly reduced in the LD condition of the higher Cd (1.0 mg/kg) group (Fig. 2). Figure 3 shows the spleen weights at the dissection. The spleen weights in the control group tended to decrease in the Sh condition compared to LD and DL condition, although they were not significantly changed. The spleen weights

in the Cd treatment group also basically did not significantly changed, except for slightly reduction in the LD condition of the higher Cd (1.0 mg/kg) group, as with the result of body weights.

The mitogen activities, however, were obviously reduced by changing the light/dark condition and by Cd treatment (Fig. 4). In LD condition, Cd had no effect on mitogen activity at lower dose (0.33 mg/kg), but had tendency to reduce the activity at higher dose (1.0 mg/kg); the absorbance decreased to about a half of control value (not significant, due to dispersion of data) (Fig. 4,

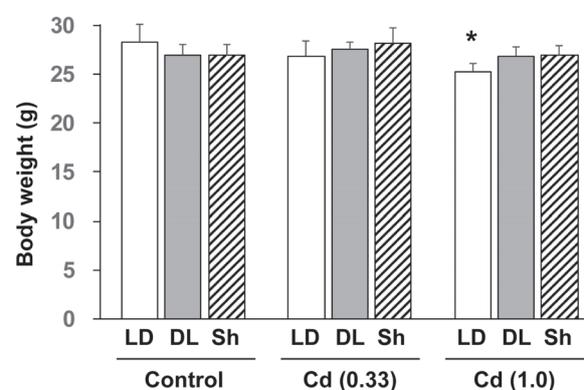


Fig. 2. Comparison of body weights (g) at the dissection. Mice were sacrificed 7 days after the last injection. LD group, white bar; DL group, gray bar; Sh group, shaded bar. \*Significantly different ( $p < 0.05$ ) from control.

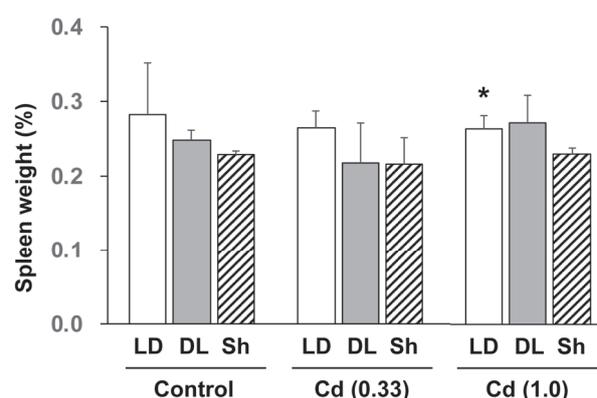


Fig. 3. Comparison of spleen weights (%) at the dissection. Mice were sacrificed 7 days after the last injection followed by removing spleens. Spleen weights were measured and expressed as percentage of body weights. LD group, white bar; DL group, gray bar; Sh group, shaded bar. \*Significantly different ( $p < 0.05$ ) from control.

white bar). Similar result was observed in DL condition (Fig. 4, gray bar). However, as shown by shaded bar in the control group, the mitogen activity of Sh condition was markedly reduced, to be about one third of LD or DL condition. Interestingly, this reduced mitogen activity was completely inhibited by higher dose of Cd (Fig. 4, shaded bar). These results indicated that each of circadian disruption and Cd exposure collectively reduced immune function as reported earlier (Blakley, 1985; Everson, 2005; Hui *et al.*, 2007; Marth *et al.*, 2001), furthermore, complex exposure, i.e., Cd exposure when being the circadian disruption, had potential to have more inhibitory effect on immune function.

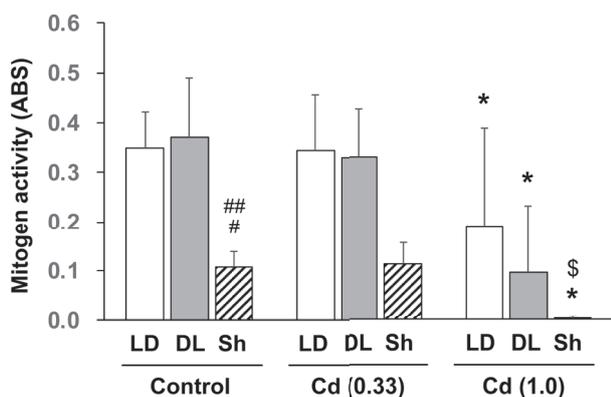
## DISCUSSION

We designed our present study to examine the combined effect of circadian disruption and Cd exposure on immune system in mice. We confirmed that the circadian disruption markedly reduced immune function estimated by mitogen activity (Fig. 4), as previously shown by many reports (Cuesta *et al.*, 2016; Geiger *et al.*, 2015). Since the circadian rhythms exist in most organs and cells, the immune system is also received circadian regu-

lation, for instance, secretions of several major cytokines, such as interleukins, interferons, and tumor necrosis factors show diurnal variation (Cuesta *et al.*, 2016). The immune system is complexes of physiological processes and act as defense to cancer cell proliferation, as well as to pathogens, like bacteria and viruses. Therefore, circadian disruption leads to immune suppressive effect, as a result, has a possibility to weaken the personal tolerance to infections and cancer proliferations.

In the modern society, the night shift work has become an indispensable working arrangement. The circadian rhythms of workers who receive LAN exposure during night shift work tend to disturb. Therefore, a long-term disturbance of circadian rhythm causes many serious health disorders, e.g., obesity (Karlsson *et al.*, 2001), type II diabetes (Morikawa *et al.*, 2005), hypertension (Esquirol *et al.*, 2011), and gastric ulcers (Knutsson and Boggild, 2010; Segawa *et al.*, 1987). Furthermore, circadian rhythm disruption also provokes several types of cancer including breast (Davis *et al.*, 2001; Schernhammer *et al.*, 2001; Stevens *et al.*, 2013), prostate (Conlon *et al.*, 2007; Kubo *et al.*, 2011; Kubo *et al.*, 2006), and colon (Kloog *et al.*, 2009; Schernhammer *et al.*, 2003). In this report, we paid attention to the combined exposure to Cd and LAN (i.e., circadian disruption), because night shift workers could be exposed to Cd through smoking tobacco and by consuming rice and wheat (Miura, 2009). Our result indicated that Cd enhanced circadian disruption-induced immune suppression (Fig. 4). As reported earlier, environmental Cd exposure may enhance the renal dysfunction caused by occupational Cd exposure (Jin *et al.*, 2004). Although Cd exposure was only short period in our experiment (4 weeks, 8 times), there is a possibility that a long-term exposure to low concentration of Cd to which persons are exposed through cigarette smoke and food intake promote the circadian disruption-induced immune suppression. Much more, the possibility may increase for the persons who work in Cd handling workplace, such as mine and smelter, as their higher environmental Cd concentration.

We previously reported the diurnal variation of Cd-induced hepatotoxicity (Miura *et al.*, 2013; Miura *et al.*, 2012) and testicular function (Ohtani *et al.*, 2013). In these reports, mice were more sensitive to Cd toxicity during light phase compared to dark phase, but our present study showed no difference in the severity of Cd-induced immune toxicity between LD and DL (Fig. 4). We think that this result may depends on experimental condition(s), i.e., not single injection with higher Cd dose but multiple injection with lower Cd, not acute experiment but sub-acute experiment with 8 injections for 4 weeks, and/



**Fig. 4.** Effect of light/dark shift condition and/or Cd treatment on mitogen activity. Male C57BL/6J mice were kept under each lightning condition (LD, DL, and Sh) and/or injected CdCl<sub>2</sub> (0.33 or 1.0 mg/kg) twice a week for 4 weeks followed by sacrificing 7 days after the last injection. The PHA-induced mitogen activity was estimated using WST-5 by measuring the absorbance (ABS) at 450 nm. LD group, white bar; DL group, gray bar; Sh group, shaded bar. \*Significantly different ( $p < 0.05$ ) from control group; #, ## Significantly different ( $p < 0.05$ ) from control LD (#) or control DL (##); \$ Significantly different ( $p < 0.05$ ) from lower Cd dose.

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or spleen (immune function) as a target organ. Additional experiments should be needed to examine the diurnal variation of Cd-induced immune function including Cd concentration in spleen and the influence to B cells as well as to T cells.

Our present data indicated the combined effect of circadian disruption and Cd on suppression of immune function, which can protect to cancer cell proliferation by T cell inactivation and has weakened the personal tolerance to infections by B cell inactivation. This result will be a basic data to consider the health maintenance not only for night shift workers but also for ordinary persons who live in environment with 24-hr society, therefore, should also be considered in public health field as well as occupational health field.

## ACKNOWLEDGMENTS

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**Conflict of interest----** The authors declare that there is no conflict of interest.

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