

## RESEARCH COMMUNICATION

# Diethylnitrosamine-induced Hepatic Lesions are Greater in Rats Maintained under a Light-dark Cycle than under Constant Light, Related to the Locomotor Activity Rhythm

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### Abstract

Environmental lighting conditions affect circadian rhythm and carcinogenesis. The effect of diethylnitrosamine (DEN, i.p., 200 mg/kg) on carcinogenesis and circadian rhythmicity under a light-dark (LD) cycle, constant dark (DD) and constant light (LL) was analyzed in rats. After the recognition of entrainment in locomotor activity rhythm to LD cycle, animals remained under the LD cycle or were released into DD or LL. Liver carcinogenicity, measured by GST-P immunostaining, was higher under the LD cycle than under DD and LL. Two weeks after DEN injection, locomotor activity in 24 hr had increased under the LD. Circadian rhythmicity might be coupled with the carcinogenicity of DEN.

**Key Words:** Carcinogenicity - light-dark cycle - circadian rhythm, locomotor activity - diethylnitrosamine

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### Introduction

Circadian clock function is coupled to cell cycle regulation (Matsuo et al., 2003), e.g., clock-gene-related DNA damage promotes cancer (Lee, 2005). A physically ablated suprachiasmatic nucleus, a center of the biological clock in mammals, mouse showed accelerated tumor progression (Filipski et al., 2003), and disrupted circadian coordination promoted malignant growth (Lee, 2005). The brain, including the suprachiasmatic nucleus, senses body information through neurons and blood-borne factors, and direct neuronal connections inform the brain about the state of various organs (Kreier et al., 2003). The suprachiasmatic nucleus is possibly involved in the rhythmic changes associated with hepatic insufficiency (Lopez et al., 2002). More than 30 years ago, Halberg et al. proposed the importance of the concept of chronotherapy of neoplasia with a background of host rhythmicity (Halberg et al., 1973); however, no guidelines have been established. Recently, it has been reported that cell proliferation (cell cycle, cancer and tumor) and circadian rhythmicity are tightly coupled, and that the loss and deregulation of PERIOD proteins is common in tumor cells (Chen-Goodspeed and Lee, 2007; Filipski et al., 2002; Lee, 2005).

Prolonged exposure to constant light, disrupting the coordination of the circadian system, has been shown to accelerate malignant process, although the precise mechanism is unclear (Filipski et al., 2005). Exposure

to constant light for several weeks suppresses the rhythms of locomotor activity and reduces activity (Filipski et al., 2005; Isobe and Nishino, 1998). A chemical carcinogen, diethylnitrosamine (DEN), induced hepatic carcinogenesis that was reported to be severe in rats kept under constant light (van den Heiligenberg et al., 1999). Meanwhile, another chemical carcinogen, dimethylbenzanthracene, induced fewer mammary tumors in rats kept under constant light than under a light-dark (LD) cycle (Anderson et al., 2000). It is appropriate to compare the lesion grade and circadian disruption against carcinogenic chemicals under alternative LD cycles, constant dark (DD) and constant light (LL) conditions. The lighting schedule modified the expression of clock genes involved in carcinogenesis (Filipski et al., 2005). Illumination is therefore a factor in the cancer risk of humans (Canaple et al., 2003; Stevens, 2006).

It is reasonable to speculate that cancer affects circadian rhythmicity, and carcinogenic drugs might affect circadian rhythm. Various nitrosamines are suspected as carcinogens in humans because intestinal bacterial flora can transform nitrites and nitrites in food into powerful carcinogenic nitrosamines (Thiery et al.; 1999). Diethylnitrosamine (DEN) was chosen to analyze the influence of circadian rhythm in the early stage of hepatocarcinogenesis. The glutathion S-transferase placental form (GST-P)-positive foci were measured as a biomarker of carcinogenesis (Moore et al. 1987; Asamoto et al., 1989; Deguchi and Pitot, 1995; Tsuda et al.; 2003).

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We therefore compared DEN-induced carcinogenic variation using GST-P immunoreactivity and locomotor activity rhythms under LD, DD and LL. Survival time was longer for hibernators than non-hibernators (Reznik et al.: 1977). It is assumed that the activity level is related to the cancer risk.

## Materials and Methods

### Animals

Thirty-one male Wistar rats (SLC, Shizuoka, Japan), 5 weeks old at the start of the experiment, were used. They were born and kept in our animal facility at room temperature, 25°C with free access to food/water and in a room under a 12 h light/dark cycle (LD cycle), with lights on at 7:00 (Zeitgeber time; ZT=0) with an intensity of 200 lx (L) and 5 lx (D), respectively. The experiment was conducted in accordance with Guidelines for Animal Experiments of Nagoya City University, Graduate School of Medical Sciences.

### Activity rhythms

Spontaneous locomotor activity rhythm was measured using an Animex (Shimadzu, Kyoto, Japan) or a running wheel cage (Natsume, Tokyo, Japan) (Isobe et al., 1992-1993; Isobe and Isobe, 1998; Isobe and Nishino, 1998). The Animex and wheel cage were set in a temperature-controlled room at 25°C. The animals were set on the wheel or Animex at 5 weeks old. After synchronizing with the LD cycle, the lighting condition was remained as the LD cycle or was changed to constant dark (DD) or constant light (LL). An intraperitoneal injection of DEN or saline was administered when animals were accustomed to each lighting condition of DD and LL for 18 and 8 days, respectively. Locomotor activity and wheel revolutions were recorded individually, and stored on disk for later analysis. The free-running circadian period of locomotor activity was estimated from the onset of activity by the eye fitness method (Pittendrigh and Daan, 1976; Isobe et al., 1992-1993; Isobe and Nishino, 1998). To calculate the free-running circadian period under DD and LL, the 8 – 18 days before DEN injection (control) and the last 10 days were used for analysis. To compare activity changes, activities in 24 hours on the day before DEN or saline injection were adjusted to 100%.

### Diethylnitrosamine injection

Diethylnitrosamine (DEN, Tokyo Chemical Industry, Tokyo, Japan) dissolved in saline for 200 mg/kg or saline was injected intraperitoneally at the indicated time of day (Figs 1, 2 and 3). The injection was performed under the LD cycle (saline, n=4; DEN, n=8), DD (5 lx, saline, n=3; DEN, n=9) or LL (200 lx, saline, n=3; DEN, n=4).

### Immunohistochemistry of Glutathione S-transferase placental form (GST-P)

Liver tissue fixed in 10% phosphate-buffered formaldehyde was processed into paraffin-embedded sections as described previously (Moore et al., 1987; Satoh et al., 1989). Liver sections of 3 mm thickness were treated with rabbit anti-rat-GST-P antibody (MBL, Nagoya,

Japan), then sequentially with the secondary antibody with the ABC method, and visualized using DAB (Hsu et al., 1990; Satoh et al., 1989). GST-P-positive liver lesion cells comprising single, 2 and more than 3 cells were measured and expressed as the number/cm<sup>2</sup> on section slides.

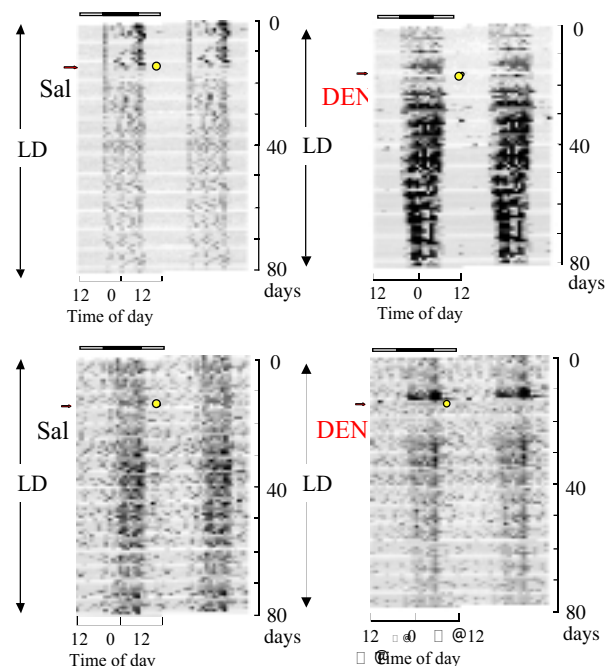
### Statistical analysis

To analyze locomotor activity rhythm, the slope of an eye-fitted line at the onset of events was used to determine the circadian period ( $\tau$ ). Differences between the means were tested by two-way analysis of variance (ANOVA) with Student's t-test, and  $p < 0.05$  indicated significance.

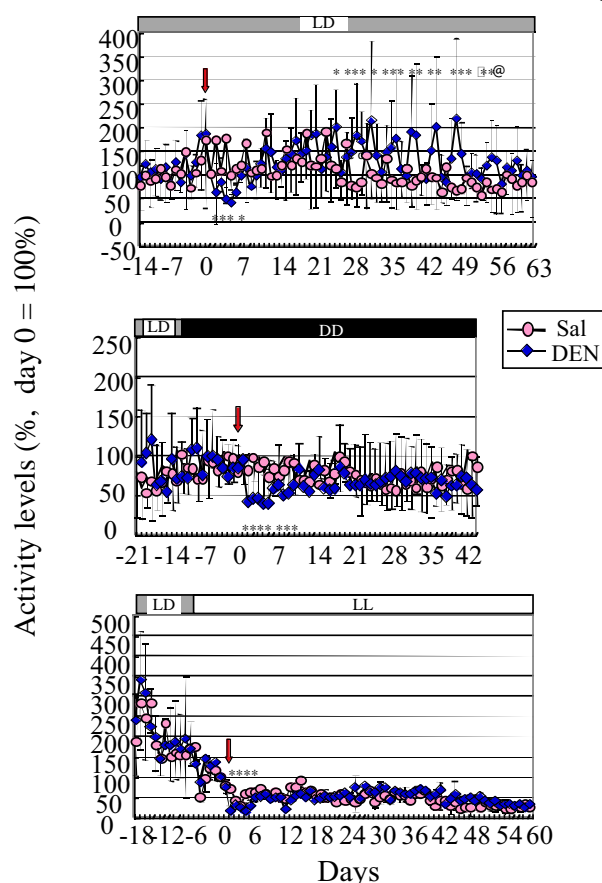
## Results

### Circadian rhythms before and after DEN injection under the LD, DD and LL

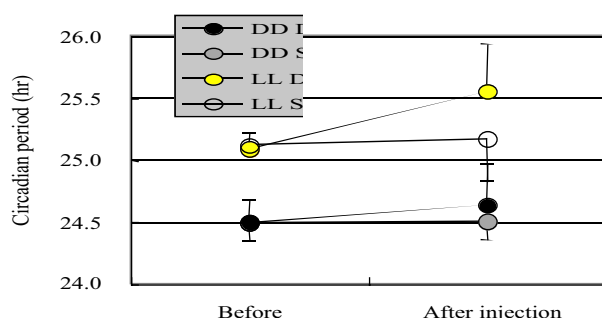
DEN injection led to a severe decrease in locomotor activity that lasted for 3 – 5 days under all lighting conditions (Figs 1, 2, 3 and 4). Saline-injected rats attained the baseline level of activity. One to two weeks after DEN injection, activities increased under the LD cycle, which was not observed in the saline-injected group (LD, Figs 1 and 4). In the saline-injected group, there was no significant difference in the level of activity 24 hr before and after the injection under all lighting conditions of LD, DD and LL (Figure 1). Taken together, the total amount of activity in 24 hrs in 2 – 7 weeks in rats treated with one-shot DEN, showed increased motor activity under LD,



**Figure 1. Effect of Diethylnitrosamine (DEN) on Locomotor Activity Rhythm under the Light-dark (LD) Cycle.** Dark spot indicates active (wheel turns or locomotion) time. Actograms were obtained by the double-plot method. Upper two actograms were obtained using the running wheel cage; the lower two using Animex. On the day indicated by a red arrow, diethylnitrosamine (DEN, 200 mg/kg, left two Figs) or saline (Sal, right two Figs) were injected at the time marked by a yellow circle. Black and white rectangles at the top indicate light and dark periods, respectively.



**Figure 2. Changes in the Levels of Locomotor Activity in the 24 hr Before and After DEN Injection under LD (top), DD (middle) and LL (bottom) Conditions.** Symbols indicate the means±SD, shown on only one side. LD: DEN, n=8 and Sal, n=4; DD: DEN, n=9 and Sal, n=3; under LL: DEN, n=4 and Sal, n=3. Activity levels the day before injection were adjusted to 100%. On the day indicated by the arrow, DEN was injected. Asterisks (\*) indicate significant (p<0.05) difference compared with the day before injection.



**Figure 3. Free-running Circadian Period under DD and LL.** Symbols indicate the means±SD, shown on only one side. Under DD: DEN, n=9 and Sal, n=3; under LL: DEN, n=4 and Sal, n=3

**Table 1. Mean Body Weights (g) ± SD**

	Saline				DEN				
	At the start	Injection†	At sacrifice§	Increase¶	At the start	Injection†	At sacrifice§	Increase¶	
LD (4)	72±15	186±12	376±49	190±32	LD (8)	70±22	173±43	362±51	189±46
DD (3)	77±6	238±25	470±65	231±42	DD (9)	73±7	226±38	424±35	198±30
LL (3)	70±2	210±14	415±35	210±15	LL (4)	72±3	220±9	381±20	161±38

At the start of experiment 5 weeks old. †: 8 - 9 week old animals. §: 8 - 9 weeks after DEN injection (17 - 18 weeks old). ¶: Body weight increase; differences between at injection and sacrifice. Numbers of animals are shown in parentheses.

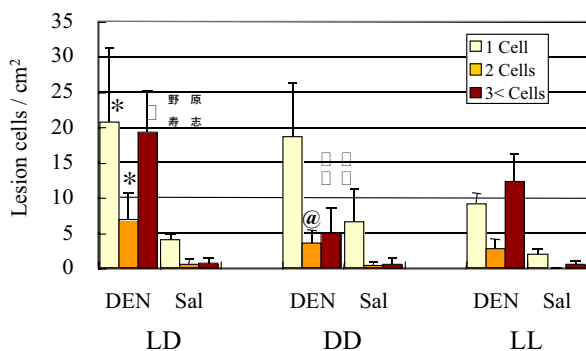
and no changes under DD and LL accompanied by no changes in the circadian period. Under the LD cycle, a significant increase in the rest period in the dark was observed, although the rat is usually active. Under LD, alpha (a, activity period, Pittendrigh and Daan, 1976) compression after DEN injection was recognized. The a-compression did not accompany the decrease of activity in 24 hrs (Figures 1 and 2); activity time was consolidated. The a-compression was not observed in control saline-injected rats using the wheel and Animex (Figure 1).

*Circadian period and phase response*

To measure the influences of DEN on the endogenous circadian oscillator, we analyzed the circadian period. The free-running circadian period under DD (5 lux) and LL (200 lux) showed similar patterns and periods to our previous report (Isobe and Isobe, 1998; Isobe and Nishino, 1998). The circadian free-running period under the DD and LL was adopted in the last ten days. Animals were sacrificed within two days after recording the activity rhythm at liver sampling to determine preneoplastic lesions. The nearest time span to the activity trait is considered to well reflect liver damage. After DEN injection, the free-running circadian rhythm continued, showing no obvious changes in period and phase under the DD and LL (Figure 3). In some animals, under LL, locomotor activity was arrhythmic 1 – 3 weeks after DEN; however, the bouts of activity were clustered, so rhythmicity was not arrhythmic, but an ultradian rhythm. The free-running circadian period was not different before and after DEN injection measured under DD (before, 24.5}0.18 hr; after, 24.6}0.33 hr) and LL (before, 25.1}0.13 hr; after, 25.6}0.38 hr) (Figure 3), and the results were not different from with saline injection. The free-running circadian period under LL was significantly longer than that under DD in both DEN- and saline-injected groups. The DEN injection time was fitted to the phase-delaying time period, and when a light pulse was given during this period (Pittendrigh and Daan, 1976), no phase shift was found under DD and LL.

*Body weight change*

Body weight and changes are shown in Table 1. Mean body weights were similar in each of the 3 groups. There was no significant difference among LD, DD and LL lighting conditions following DEN injection. Body weight increase in DEN- and saline-treated rats was 189±46 and 190±32 g under the LD cycle, 198±30 and 231±42 g under DD, and 161±38 and 210±15 g under LL, respectively. The increase in body weight gain was slightly greater under LD and DD in the DEN-injected group (Table 1).



**Figure 4. Numbers of GST-P-positive Foci.** GST-P-positive foci were classified to a single cell, 2- cell and more than 3 cell lesions. Data are the means $\pm$ SD. \*, Significantly higher than under LL,  $p < 0.05$ . @ and @@ indicate significantly lower than under LD,  $p < 0.05$  and  $p < 0.01$ , respectively

#### GST-P immunoreactivity

The lesion grade analyzed by GST-P immunoreactivity was comparable when DEN was injected under the LD cycle, DD and LL to each saline-injected group. Quantitative data for GST-P-positive foci, at 8 weeks after DEN injection, are summarized in Figure 4. The number of foci of more than three cells, which closely reflects the neoplastic lesion grade, was significantly greater in rats kept under the LD cycle. The number of foci classified as single, two and more than three cells was well correlated in the liver of each specimen. The correlation coefficient between a single cell with 2 cells, a single cell with more than 3 cells, and two cells with more than 3 cells was 0.727 ( $n=23$ ,  $p < 0.01$ ), 0.828 ( $n=23$ ,  $p < 0.01$ ), and 0.767 ( $n=23$ ,  $p < 0.01$ ), respectively.

In addition, we analyzed the correlation of the lesioned foci number in more than 3 cells with activity levels in 24 hours, with the ratio of activity during light and dark periods in each lighting condition; however, we failed to detect any significant correlation.

## Discussion

Carcinogenicity, which was quantitatively analyzed by GST-P staining, was higher in the LD group than in DD and LL groups in our results (Figure 4). The extent of DEN-induced neoplastic lesions differed from the previous report (van den Heiligenberg et al., 1999). When DEN was administered daily in drinking water, carcinogenic activity was reported to be higher in rats kept under constant light than under the light-dark cycle (van den Heiligenberg et al., 1999). The reason is unclear; however, the DEN administration route in previous reports, given in drinking water, differed from our i.p. injection. Moreover, the pathological determination method was different from the previous study that used hematein-eosin-saffran staining, while we adopted the more sensitive early-detection GST-P immunostaining method (Moore et al., 1987; Satoh et al., 1989; Tsuda et al., 2003).

The higher carcinogenicity under the LD cycle might be explained in relation to corticosterone (van den Heiligenberg et al., 1999; Isobe and Isobe, 1998). Corticosterone rhythmicity is lost and melatonin release

is suppressed under the constant light condition. In another report, under DEN-induced carcinogenic conditions, corticoid injection inhibited the selective growth of pre-cancerous cells (Bouzahzah et al., 1998). Corticosterone activity, which normally synchronizes cell proliferation, temporarily inhibits DNA synthesis in a circadian manner (Barbason et al., 1995). Our results of low carcinogenicity under LL might indicate that the increase of corticosterone is related with the inhibition of carcinogenesis.

Under free-running conditions, photic stimulation usually induces phase shift (Pittendrigh and Daan, 1976). Phase shift was not detected after DEN injection, even when the injection time corresponded to the phase-delaying time period, early subjective nighttime and late subjective daytime against the light pulse (Pittendrigh and Daan, 1976). This indicates that DEN does not directly affect the pacemaker itself, or affects it in a different manner than the light pulse to the pacemaker.

It is apparent that illumination control is a strong factor against carcinogenicity. In humans, the breast cancer risk is higher in modern society, with longer working time under illuminated conditions as a factor in circadian disruption (Stevens, 2006). Tumor growth factor was high in jet-lagged (shift of LD cycle) mice compared with control LD mice, whereas exposure to constant light or darkness had no effect (Filipsiki et al. 2005).

There is increasing evidence that melatonin administration increases not only longevity but also inhibits DEN-induced carcinogenesis (Anisimov et al., 2006). In nocturnal rats, melatonin synthesis and release is higher during the dark period, which corresponds to the active period (Isobe et al., 2001; Isobe and Nishino, 2004). The pattern of entrainment of locomotor activity rhythm to the photoperiod is highly correlated with the pattern of melatonin release (Klante et al., 1999). In this result, lesion size and numbers were higher in the LD group than in DD and LL groups. On the other hand, blind women suffer more breast cancer than sighted women, which is independent of nocturnal melatonin release (Stevens, 2006). Therefore, from our results it is proposed that the anti-oxidative effect of melatonin as a defensive factor against tumor and cancer would be overridden by the non-decrease of locomotor activity during the nocturnal period under the LD cycle. The tumor suppression effect of melatonin might be limited by the experimental design.

The feeding schedule is tightly coupled with clock-controlled gene expression and carcinogenesis (Canaple et al., 2003; Filipinski et al., 2005; Oishi et al., 2004). Body weight gain in the DEN-injected group compared with the saline-injected group showed a greater tendency in LD than in LL (Table 1), similar to the previous report (van den Heiligenberg et al., 1999). The results that the extent of activity increase and the tendency toward greater body weight increase in the LD cycle than under the LL suggest that food should be increased. Increased food intake and activity enhanced the development of hepatocellular carcinoma by DEN (Petruska et al., 2001). This is directly reflected by food intake; more food intake would increase the tumor size and lesion number (Ha et al., 2001).

Under LD conditions, circadian locomotor activity rhythm during darkness is consistent with the light-sensitive parts of both E (evening) and M (morning) oscillators. The a-compression was caused by the delay of activity onset (driven by E oscillator) and advance of activity offset (driven by M oscillator). It was expected that individual variation in coupling strength between two oscillators (E and M) would display temporal a-compression (Pittendrigh and Daan, 1976). The a-compression accompanying the increase of locomotor activity might be related with higher carcinogenicity (Steinlechner et al., 2002; Lee, 2005; Cavadini et al., 2007; Chen-Goodspeed and Lee., 2007). In the Filipinski study, the exposure of mice to constant darkness or light exerted no effect on tumor growth (Filipinski et al., 2002). Concerning activity compression, spontaneously hypertensive rats (SHR) showed rigid rhythmicity, and shortened activity time (a-compression) compared to control WKY, whose ancestral origin is the same as SHR (Isobe et al., 1992-1993). SHR were entrained to the LD cycle more slowly than WKY (Peters et al., 1994), and were more susceptible to DEN-induced carcinogenicity than control Wistar rats (Asamoto et al., 1989). It is suspected that the rigidity of the activity pattern and increase of activity are related with the degree of carcinogenicity.

Before and after DEN administration, the circadian period under free-running conditions in DD and LL did not significantly vary. Temporal restricted feeding under the LD cycle and DD changed peripheral oscillators, while cyclic clock gene expression in the SCN was not altered (Damiola et al., 2000). The DEN carcinogenic agent causes cancer and changes locomotor activity rhythm; however, the central clock might not be affected. If we modified the DEN administration method or increased the recording period, we could find some differences.

The locomotor activity level correlated with the extent of cancer damage; hibernating hamsters tolerated a higher dose of DEN and developed fewer neoplasms (Reznik et al., 1977). A longer non-active period would reduce the cancer risk (Cavadini et al., 2007; Filipinski et al., 2004), and a lower activity level under LL than LD and DD would be related with fewer lesion foci.

The carcinogenicity of DEN was more potent under the light-dark cycle than under constant dark and light. The circadian rhythm of activity in relation to the lighting condition might be related with carcinogenicity. provides some important information about dentists' in the dental clinic setting.

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