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Chronopharmacology of melatonin in mice to maximize the antitumor effect and minimize the rhythm disturbance effect

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MLT, melatonin;

SCN, suprachiasmatic nucleus;

S-180, sarcoma 180;

AUC, area under the concentration-time curve;

MRT, mean residence time

Abstract

The influence of dosing time on the antitumor effect and the rhythm disturbance effect of melatonin (MLT) was investigated in ICR male mice under a light-dark (12:12) cycle. In tumor-bearing mice, the antitumor effect of MLT (1 mg/kg, intraperitoneal) was most effective in the dark phase; and the rhythm disturbance effect of MLT on the locomotor activity was more serious in the light phase than in the dark phase. The antitumor effect and the rhythm disturbance effect of MLT increased when the specific binding of MLT receptor in target tissues, tumor or suprachiasmatic nucleus (SCN), increased and they decreased when the level decreased. Furthermore, since luzindole (LZD), a both MT_1 and MT_2 blocker, caused the antitumor effect or rhythm disturbance effect of MLT to decrease, it is suggested that the time-dependent change of the pharmacological effects of MLT were influenced by that of MLT receptor(s) function. On the other hand, there was no significant dosing time-dependent change of MLT concentration in tumor or brain after injection. Thus, the pharmacokinetic factor does not appear to contribute to the dosing

time-dependent effect of MLT. These results suggest that by choosing the most suitable dosing time for MLT, the efficacy of the drug can be increased and the toxicity of the drug can be decreased in certain experimental and clinical situations.

A large number of rhythmic variables are influenced by environmental factors such as light, temperature, and social communication that vary cyclically in nature and serve to synchronize biological rhythms to the daily rotation of the earth. Responses to a variety of drugs show 24-hr rhythmicity (Ohdo et al., 1991, 1996, 1998; Watanabe et al., 1992). Use of a chronopharmacological strategy can improve the outcome of pharmacotherapy. However, several drugs cause alterations in the 24-hr rhythms of biological, physiological and behavioral processes (Duncan, 1996; Horikawa et al., 2000; Ohdo et al., 2001). The alteration of rhythmicity is sometimes associated with therapeutic effects, or may lead to illness and altered homeostatic regulation.

MLT is an indoleamine synthesized from serotonin in the pineal gland. MLT has many pharmacological and physiological effects. One of them is the antitumor effect. Both in vitro and in vivo, MLT has been found to inhibit neoplastic growth and to delay tumor progression (Cini et al., 1998). Moreover, MLT is well known to show no toxic effects (Lissoni et al., 1993a, 1993b), and, therefore, as a non-toxic anticancer agent. In phase II-type clinical trials, the effectiveness of MLT was shown either alone or in combination with other chemotherapeutic drugs, as an anticancer agent (Panzer and

Viljoen, 1997). Although very little is known about the exact mechanisms by which MLT inhibits tumor growth, it has been shown that MLT has both a direct oncostatic effect on tumor cells (Blask, 1993, 1997) and an anticancer action via stimulation of the immune system (Conti and Maestroni, 1995). It was suggested that both direct and indirect antitumor effects are via its specific binding site in tumor cells or immune cells. Some drugs, which elicit their actions via their specific binding sites, have been shown to have a dosing time-dependent effect in relation to their receptor sensitivity (Takane et al., 2000). Therefore, it is probable that the antitumor effect of MLT changes according to the time of administration. However, the influence of MLT dosing time on tumor growth has not been revealed.

MLT has an effect on biological rhythms similar to light. The effects of MLT treatment or light exposure on biological rhythms follow a phase-response curve. Light exposure in the late dark phase causes a phase advance in the sleep-wake cycle, while light exposure in the early dark phase causes a phase delay. However, MLT treatment produces the opposite effects on biological rhythms to light (Lewy et al., 1992). Namely, MLT treatment in the late light phase causes a phase advance in

the sleep-wake cycle, while MLT treatment in the early light phase causes a phase delay. This effect of MLT appears to act via the SCN, the principal circadian clock. Due to this effect, in clinical situations, MLT is used to cure sleep disorders, jet lag and so on. However, a low dose of MLT (0.1-10 mg) given in the middle of light phase when endogenous levels are lowest causes decreased sleep-onset latency, decreased oral temperature and a reduction in the number of correct responses on the Wilkinson auditory vigilance task (Dollins et al., 1993). Therefore, if dosing time is not appropriate, this effect of MLT may cause rhythm disorders.

The purpose of this study was to investigate the influence of MLT dosing time on tumor growth in tumor-bearing mice to maximize the antitumor effect and to investigate the influence of MLT dosing time on the locomotor activity to minimize the risk of rhythm disturbance caused by exogenous MLT. The mechanism underlying the dosing time-dependent difference was elucidated from the viewpoints of MLT pharmacodynamics or pharmacokinetics.

Methods

Animals and cell culture. Four-week-old male ICR mice (Charles River Japan, Inc., Kanagawa, Japan) were housed 3-10 per cage under standardized light-dark cycle conditions (lights on at 07:00 off at 19:00) at a room temperature of 24 ± 1°C and humidity of 60 ± 10% with food and water ad libitum. All mice were adapted to their light-dark cycle for 2 weeks prior to experiments. Sarcoma 180 (S-180) cells (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan), were grown in eagle's minimal essential medium supplemented with 10% FBS, 0.05% kanamycin, penicillin and streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. S-180 cells were transplanted with $5x10^5$ cells into the hind footpads of mice at the day before drug injection.

Preparation of dosing solutions. MLT (Wako Pure Chemical Industries Ltd., Osaka, Japan) dissolved in saline to yield an appropriate concentration of 0.1 mg/ml. Luzindole (LZD, 2-benzyl-N-acetyltryptamine) (Wako Pure Chemical Industries Ltd., Osaka, Japan) was prepared in 50% ethanol and then diluted in Krebs' solution to

appropriate concentrations. The final ethanol concentrations in the tissue bath did not affect contractility. MLT and LZD were used at an intraperitoneal (ip) dose of 1 mg/kg or 10 mg/kg by means of 23-gauge needle connected to a 1-ml syringe. The volume of drug solutions administered was 10 ml/kg. Other reagents, purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), were of analytical grade and used without further purification.

Influence of MLT dosing time on growth of tumor weight. Firstly, groups of 9-10 mice were intraperitoneally injected with MLT (1 mg/kg) at 07:00, 13:00, 19:00 or 01:00 to investigate the influence of dosing time on the antitumor effect of MLT. Secondly, we selected two different dosing times such as peak and trough. Groups of 9-10 mice were intraperitoneally injected with MLT (1 mg/kg), MLT and LZD (10 mg/kg) or saline at 13:00 or 01:00. Drug or saline was administered daily for 8 days. On the 8th day, the growth of S-180 cells was monitored by calculating the tumor volume from caliper measurements. The tumor weight was calculated according to the following equation: Tumor weight = $AxB^2/2$. where A is the longer diameter and B is the shorter one (mm).

Influence of MLT dosing time on locomotor activity. After groups of 3 mice were intraperitoneally injected with saline for 5 days, they were intraperitoneally injected with MLT (1 mg/kg), MLT and LZD (10 mg/kg) or saline at 13:00 or 01:00. Drugs were administered daily for 7 days. Locomotor activity was measured using scanet SV-10LD (Toyo Sangyo Co. Ltd., Toyama, Japan), and the activity count (number of movements) was recorded at 1 hr intervals. For visualization of locomotor activity rhythm, hourly the activity counts were calculated using a moving average with a 4-hr window. The average counts were plotted at 09:00, 13:00, 17:00, 21:00, 01:00 or 05:00.

Time-dependent change in specific binding of MLT to tumor or SCN cells. Tumor tissues were removed from 6 mice at 07:00, 13:00, 19:00 or 01:00. To obtain the three SCN samples, coronal brain slice were prepared from 18 mice using rodent brain matrix (RBM-2000C; ASI Instruments Inc., Warren, Michigan, USA) at 13:00 or 01:00. Tumor or SCN was homogenized in 1 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4). Homogenates were then centrifuged at 15,000 rpm for 15 min at 4°C. The obtained pellet was resuspended in 4 or 1 (tumor 4, SCN 1) ml of Tris-HCl buffer (pH

7.4). After that, homogenates were centrifuged again. Pellets were resuspended in 3 or 1 (tumor 3, SCN 1) ml of Tris-HCl buffer. The protein concentration was approximately 3 or 1 (tumor 3, SCN 1) mg/ml using Lowry's method (DC Protein Assay; Bio-Rad, Hercules, CA, USA). The binding assay was done in the method already described (Takane et al., 2000). The binding assay was performed with a reaction mixture (total volume, 200 μl) containing 100 μl of aliquot of tumor homogenate and 50 nM [*O-methyl*-^{3}H] MLT (Amersham Pharmacia Biotech Ltd, Little Chalfont, Buckinghamshire, UK) or containing 100 μl of aliquot of SCN homogenate and 12.5 to 50 nM [*O-methyl*-^{3}H] MLT. Non-specific binding was determined in the presence of 100 μM MLT. After incubation at 4°C for 5 hr, the reaction mixture together with 100 μl of Tris-HCl buffer for washing the tube was laid over the 300 μl ice-cold FBS, and centrifuged at 10,000 rpm for 3 min at 4°C. The supernatant was removed, and then the pellet transferred to scintillation vials with 10 ml of scintillation cocktail and counted using a liquid scintillation counter (LSC-1000; Aloka Co., Mitaka, Tokyo Japan) after keeping for 6 hr. Specific binding is the difference between binding determined in the absence of ligand and in the presence of ligand and was calculated

as follows: Specific binding [fmol/mg protein] = {(Total binding [fmol/mg protein])-(Non-specific binding [fmol/mg protein])}. The data were plotted according to the method of Scatchard. The number of MLT receptors and the dissociation constant (Kd) were calculated by the method of least squares to fit the data and this experiment went three times.

Influence of MLT dosing time on pharmacokinetics. Groups of 6 mice were intraperitoneally injected with 1 mg/kg of melatonin at 07:00, 13:00, 19:00 or 01:00. Blood samples (approximately 50 μl for each sample) were drawn by orbital sinus collection using micropipettes at 10, 30, 60 or 120 min after MLT injection. Blood samples were immediately centrifuged at 3,000 rpm for 3 min. A mixture of plasma sample (20 μl), internal standard (3,4-dimethoxybenzoic acid; veratric acid, 10 mg/ml, 20 μl) and methanol (400 μl) was mixed with an automatic mixer (S-100, Taitec, Saitama, Japan) for 20 sec and centrifuged at 10,000 rpm for 20 min to deproteinate the samples. The supernatant was evaporated on a Speed Vac Plus SC110A (Savant Instruments. Inc. Farmingdale, NY, USA) for 120 min. The residue was dissolved in 30 μl of methanol. The insoluble substance was removed by centrifugation at 10,000

rpm for 3 min. Then, 20 μl of the solution was injected into the HPLC system which comprised a pump (LC-10AD Liquid Chromatograph, Shimadzu, Kyoto, Japan), a detector (RF-10A Spectrofluorometric Detector, Shimadzu), a chromatopac (C-R1B, Shimadzu), a guard column (TSK-GEL ODS-80TS, 5 mm, 3.5 mm I.D.x15 mm, Toyo Soda, Tokyo, Japan) and an analytical column (TSK-GEL ODS-80TS, 5 mm, 4.6 mm I.D.x15 mm, Toyo soda). The mobile phases consisted of 0.01 M acetate buffer (pH 4.25) solution and methanol (75:25, v/v). The peak areas were integrated using a data processor. Tumor or brain was removed at 30 min after MLT injection and homogenized in 1 ml of methanol. The homogenate was centrifuged at 15,000 rpm for 15 min. The sample was prepared and determined using HPLC in the same way as the blood samples described above.

Statistical analysis. The statistical significance of differences between groups was validated by analysis of variance (ANOVA), Tukey's test and Student's t test. Statistical moment analysis was used to calculate the pharmacokinetic parameters such as area under the time-concentration curve (AUC) and mean residence time (MRT). A probability level of $P<0.05$ was considered to be significant.

Results

Influence of MLT dosing time on growth of tumor weight. The influence of MLT (1 mg/kg) dosing time (07:00, 13:00, 19:00 or 01:00) on tumor growth is shown in figure 1A. The tumor weight in mice injected with MLT at 07:00 or 01:00 was significantly suppressed compared with that in mice injected with MLT at 13:00 (07:00 $P<0.05$, 01:00 $P<0.01$). The tumor weight in mice injected with MLT at 19:00 showed a tendency to be suppressed by MLT. Moreover, the influence of LZD (10 mg/kg) dosing on the antitumor effect of MLT is shown in Figure 1B. The tumor weight in mice injected with MLT at 13:00 was not significantly different from that in control mice and LZD did not affect the antitumor effect of MLT at 13:00. On the other hand, the tumor weight in mice injected with MLT at 01:00 was significantly lower than that in control mice ($P<0.05$), and LZD significantly blocked the antitumor effect of MLT at 01:00 ($P<0.05$).

Time-dependent change in specific binding of MLT to tumor cells. The specific binding of MLT to tumor cells is shown in Figure 2. The specific binding of MLT to

tumor cells prepared at 13:00 showed a minimum value. The specific binding of MLT to tumor cells prepared at 01:00 showed the maximum value and was significantly higher than that prepared at 07:00, 13:00 or 19:00 (07:00, 19:00 $P<0.05$, 13:00 $P<0.01$).

Influence of MLT dosing time on locomotor activity. The time course of the locomotor activity in mice after daily MLT treatment is shown in Figure 3A and 3B. The locomotor activity in mice injected with MLT at 13:00 was suppressed immediately after the initiation of MLT treatment. On the other hand, the locomotor activity in mice injected with MLT at 01:00 was gradually decreasing each day and finally, on day 5 after initiation of drug treatment, reached to the same level as mice injected with MLT at 13:00. LZD (10 mg/kg) blocked the rhythm disturbance effect of MLT at both 13:00 and 01:00, and the locomotor activity in mice injected with LZD and MLT at 13:00 or 01:00 was not significantly different from that in control mice injected with saline.

Time-dependent change in specific binding of MLT to SCN cells. The specific binding of MLT to SCN cells is shown in Figure 4. The specific binding data was replotted by the method of Scatchard. The parameter calculated from the Scatchard

analysis is shown in Table 1. The specific binding of MLT to SCN cells prepared at 13:00 was significantly larger than that prepared at 01:00 ($P<0.01$). The apparent Kd value did not differ significantly between SCN cells prepared at 13:00 and at 01:00.

Influence of MLT dosing time on MLT pharmacokinetics and MLT concentration in plasma, tumor or brain. The time course of plasma MLT concentrations in mice injected with MLT at 07:00, 13:00, 19:00 or 01:00 is shown in Table 2 and their pharmacokinetic parameters are shown in Table 3. The plasma MLT concentration at 30 min after the drug injection was significantly different among mice injected with MLT at 07:00, 13:00, 19:00 or 01:00 ($P<0.05$). The maximum or minimum plasma MLT concentration was observed when MLT was injected at 01:00 or 19:00. AUC, CL and Vd were significantly different among them (AUC, CL; $P<0.01$, Vd; $P<0.05$). The maximum or minimum AUC was observed when MLT was injected at 07:00 or 13:00. The maximum or minimum CL was observed when MLT was injected at 13:00 or 07:00. The maximum or minimum Vd was observed when MLT was injected at 13:00 or 07:00. However, there was no significant difference in MRT or Ke among them. MLT concentrations in tumors at 30 min after the drug injection at

07:00, 13:00, 19:00 or 01:00 are shown in Figure 5. MLT concentrations did not differ significantly among mice injected with the drug at 07:00, 13:00, 19:00 or 01:00. The maximum or minimum MLT concentration in tumors was observed when the drug was injected at 19:00 or 13:00. MLT concentrations in the brain at 30 min after the drug injection at 13:00 or 01:00 are shown in Figure 6. MLT concentrations did not differ significantly between mice injected with the drug at 13:00 and at 01:00.

Discussion

MLT has a capacity to inhibit the proliferation of various tumor cell lines. The antitumor effect of MLT to S180 cells was reported both *in vitro* (Sze et al., 1993) and *in vivo* (Li and Xu, 1997). In the present study, the growth of S180 implanted in mice was significantly inhibited by MLT. Furthermore, the antitumor effect of MLT was significantly different according to dosing time. Namely, the antitumor effect of MLT was observed in mice injected with MLT at 07:00, 19:00 or 01:00, but not at 13:00.

MLT exerts its proposed antitumor action through direct cellular effects and immune stimulatory effects. MLT's direct antitumor action was suggested to be through its receptor(s) MT_1 and/or MT_2 leading to receptor activation and association with an inhibitory G protein (Blask et al., 1999). In the present study, since LZD, both an MT_1 and MT_2 blocker, inhibited MLT's antitumor effect, MLT's antitumor effect was suggested to be influenced by the MLT receptor(s) function. Moreover, the specific binding of MLT to tumor cells showed rhythmicity with the minimum value in cells prepared at 13:00 when the antitumor effect of MLT was not observed. In general, the

rhythm of MLT receptor(s) density is affected by the rhythm of the MLT level in blood. Namely, prolonged exposure in high concentration to MLT causes MLT receptor(s) desensitization, and short time exposure at a low concentration of MLT does not cause MLT receptor(s) desensitization. In addition, it was reported that MLT directly suppresses the growth of cells by delaying their progression from the G1 phase to S phase of the cell cycle (Cos et al., 1991; Shiu et al., 1999; El-Missiry and Abd El-Aziz, 2000). In addition, the middle of the light phase is the time that S180 implanted in mice has the highest percentage of cells in the later S phase until the beginning of the G2 phase (Akagi et al., 2003). Although it is not known whether the rhythm of the MLT concentration influences a cell cycle, it is suggested that the time-dependent change of MLT antitumor effects is related to the change in the MLT receptor in tumor cells.

Furthermore, MLT exerts indirect antitumor effects such as the immune stimulatory effect mediated by MLT. MLT enhances the activity of tumoricidal cells and potentiates the production and release of some cytokines, TNF, IL-2 and IFNγ (Neri et al., 1995). MLT injection in the late light phase, but not in the early light phase, increases the serum IFNγ level (Champney et al., 1998). The time-dependent effects of MLT on the

immune system may contribute to time-dependent antitumor effects. Certainly, the specific binding sites of MLT are identified on immune cells (Calvo et al., 1995) and their expression may also show the 24-hr rhythm.

We also examined the influence of MLT dosing time on sleep-wake rhythm, because MLT has the effect on biological rhythms, notably sleep-wake rhythm. If MLT dosing time is not appropriate, MLT might cause the rhythm disturbance as a side effect of MLT being a non-toxic anticancer agent. We chose 13:00 when the antitumor effect of MLT was not observed, and 01:00 when the effect was the highest. The effect of MLT on locomotor activity varied depending on dosing-time. The altered locomotor activity induced by MLT was more potent in mice injected with the drug at 13:00 than at 01:00.

The effect of MLT on 24-hr rhythm of locomotor activity appears to be mediated by the direct action of MLT on the SCN, since MLT shifts the rhythmicity of electrical activity in the rodent SCN *in vitro* (McArthur et al., 1991, 1997; Starkey et al., 1995; Starkey, 1996). MLT appears to evoke its effects on SCN function through the G protein receptors with high affinity (Reppert and Weaver, 1995). In the present study,

since LZD inhibited MLT's rhythm disturbance effect, MLT's rhythm disturbance effect was suggested to be decided by the MLT receptor(s) function. Moreover, the specific binding of MLT was significantly higher for SCN cells prepared at 13:00 than that prepared at 01:00. This result supports findings of other studies in which the number of MLT binding sites in the rat SCN showed a significant 24-hr rhythm with higher levels in the light phase (Vacas and Cardinali, 1979; Gauer et al., 1993; Tenn and Niles, 1993). In addition, not only the exposure to MLT but also the photoperiod plays an important role in regulating MLT receptor(s) density in SCN cells (Masson-Pevet et al., 2000). 2-[^{125}I]-indolmelatonin binding in the SCN increases in pinealectomized rats exposed to a one-hour light pulse during the night compared with rats kept in constant darkness. Namely, the time-dependent function of MLT receptor corresponded well to the finding that the rhythm disturbance effect of MLT on locomotor activity was more serious in mice injected with the drug at 13:00 than 01:00. Therefore, one of the reasons why the locomotor activity in mice injected with the drug at 01:00 decreased over time although the activity in mice injected with the drug at 13:00 was immediately decreased might be the difference of the function in MLT

receptor (i.e. resolution and saturation in MLT receptor) between 01:00 and 13:00. In addition, other reasons might be related to the hypnotic effect relevant to the function of MLT receptor since the hypnotic effect of MLT is stronger when MLT acts during the rest period (13:00) than during the active period (01:00). Moreover, an inverse relation between the melatonin binding sites in the tumor preparations and brain section was observed. The reason for this phenomenon might be that tumor cells are not controlled more strongly than SCN cells by light.

The AUC of MLT in plasma showed a significant dosing time-dependent difference with a maximum value at 07:00 and minimum value at 13:00. The 24-hr rhythm of blood flow in eliminative organs partially influences the time-dependent change of drug pharmacokinetics (Labrecque et al., 1988). On the other hand, no significant difference was observed between the liver blood flows in the light phase and the dark phase in rats (Hori et al., 1996). However, the highest CL of MLT was observed during the middle of the light phase. The *in vivo* metabolism of the pineal hormone MLT consists mainly of 6-hydroxylation followed by sulphate or glucuronide conjugation (Arendt, 1988). In humans, 6-hydroxylation is mainly, but not exclusively, caused by

CYP1A2, the high-affinity enzyme involved in MLT metabolism (Facciola et al., 2001). However, it is not clear which enzyme participates in MLT metabolism in mice. MLT metabolic enzyme activity may have a 24-hr rhythm with high activity during the middle of the light phase. On the other hand, the MLT concentration in the tumor or brain tissues at 30 min after MLT injection did not differ significantly among mice injected with MLT at each time although these concentrations are much higher than endogenous MLT level. Therefore, the pharmacokinetics of MLT does not appear to contribute to the dosing time-dependent change in the antitumor effect and the rhythm disturbance effect of MLT.

The findings of the present study suggest that there is a dosing time-dependent change in the antitumor effect and the rhythm disturbance effect of MLT. So, when we choose the dosing time of MLT, consideration of both the influence of biological rhythms on the pharmacological action of MLT and the influence of MLT on biological rhythms are required. Moreover, it is suggested that dosing time-dependent changes of MLT's effects are caused by time-dependent differences in the sensitivity particularly at the receptor level of tumor cells or SCN cells to MLT, respectively.

Therefore, the choice of dosing time based on the 24-hr rhythm of MLT receptor expression in tumor cells and SCN cells may help establish a rational chronotherapeutic strategy, increasing the antitumor activity of the drug and decreasing the risk of the rhythm disturbance caused by MLT in certain clinical situations.

References

Akagi T, Ushinohama K, Kage Y, Ishizaki T, Makinosumi T, Yamauchi A, Taguchi Y, Inoue K, Yukawa E, Higuchi S and Ohdo S (2003) Cell kinetics-dependent antitumor effect of irinotecan hydrochloride induced by the synchronizing effect of hydroxyurea: cell kinetics and dosing time. *Life Sci.* **72**:1183-1197.

Arendt J (1988) Melatonin. *Clinical Endocrinology* **29:**205-229

Blask DE (1993) Melatonin in oncology; in Yu H-S and Reiter RJ (eds): Melatonin. Biosynthesis, physiological effects, and clinical applications. Boca Raton, CRC Press**:**447-477

Blask DE (1997) Systemic, cellular and molecular aspects of melatonin action on experimental breast carcinogenesis; in Stevens RG, Wilson BW and Anderson LE (eds): The Melatonin hypothesis. Breast cancer and the use of electric power. Columbus, Battelle Press**:**257-275

Blask DE, Sauer LA, Dauchy RT, Holowachuk EW, Ruhoff MS and Kopff HS (1999) Melatonin inhibition of cancer growth in vivo involves suppression of tumor fatty

acid metabolism via melatonin receptor-mediated signal transduction events. *Cancer Res* **59:**4693-4701

Calvo JR, Rafii-el-Idrissi M, Pozo D and Guerrero JM (1995) Immunomodulatory role of melatonin: Specific binding sites in human and rodent lymphoid cells. *J Pineal Res* **18:**119-126

Champney TH, Allen GC, Zannelli M and Beausang LA (1998) Time-dependent effects of melatonin on immune measurements in male Syrian hamsters. *J Pineal Res* **25:**142-146

Cini G, Coronnello M, Mini E and Neri B (1998) Melatonin's growth-inhibitory effect on hepatoma AH 130 in the rat. *Cancer Lett* **125:**51-59

Conti A and Maestroni GJM (1995) The clinical neuroimmunotherapeutic role of melatonin in oncology. *J Pineal Res* **19:**103-110

Cos S, Blask DE, Lemus-Wilson A and Hill AB (1991) Effects of melatonin on the cell cycle kinetics and "estrogen-rescue" of MCF-7 human breast cancer cells in culture. *J Pineal Res* **10:**36-42

Dollins AB, Lynch HJ, Wurtman RJ, Deng MH, Kischka KU, Gleason RE and

Lieberman HR (1993) Effect of pharmacological daytime doses of melatonin on human mood and performance. *Psychopharmacol* **112:**490-496

Duncan WC (1996) Circadian rhythms and the pharmacology of affective illness. *Pharmacol Ther* **71:** 253-312

El-Missiry MA and Abd El-Aziz AF (2000) Influence of melatonin on proliferation and antioxidant system in Ehrlich ascites carcinoma cells. *Cancer Lett* **151:**119-125

Facciola G, Hidestrand M, von Bahr C and Tybring G (2001) Cytochrome P450 isoforms involved in melatonin metabolism in human liver microsomes. *Eur J Clin Pharmacol* **56:**881-888

Gauer F, Pevet MM, Skene DJ, Roles BV and Pevet P (1993) Daily rhythm of melatonin binding sites in the rat pars tuberalis and suprachiasmatic nuclei; Evidence for a regulation of melatonin receptors by melatonin itself. *Neuroendocrinol* **57:**120-126

Hori K, Zhang QH, Li HC, Saito S and Sato Y (1996) Timing of cancer chemotherapy based on circadian variations in tumor tissue blood flow. *Int J Cancer* **65:**360-364

Horikawa K, Yokota S, Fuji K, Akiyama M, Moriya T, Okamura H and Shibata S (2000)

Nonphotic entrainment by 5-HT1A/7 receptor agonists accompanied by reduced Per1 and Per2 mRNA levels in the suprachiasmatic nuclei. J *Neurosci* **20:**5867-5873

Labrecque G, Belanger PM, Dore F and Lalande M (1988) 24-hour variations in the distribution of labeled microspheres to the intestine, liver and kidneys. *Ann Rev Chronopharmacol* **5:**445-448

Lewy AJ, Ahmed S, Jackson JM and Sack RL (1992) Melatonin shifts human circadian rhythms according to a phase-response curve. *Chronobiol Int* **9:**380-392

Li JC and Xu F (1997) Influences of light-dark shifting on the immune system, tumor growth and life span of rats, mice and fruit flies as well as on the counteraction of melatonin. *Biol Signals* **6:**77-89

Lissoni P, Barni S, Tancini G, Ardizzoia A, Rovelli F, Cazzaniga M, Brivio F, Piperno A, Aldeghi R, Fossati D, Characjeius D, Kothari L, Conti A and Maestroni GJM (1993a) Immunotherapy with subcutaneous low-dose interleukin-2 and the pineal indole melatonin as a new effective therapy in advanced cancer of the digestive tract. *Br J Cancer* **67:**1404-1407

Lissoni P, Barni S, Tancini G, Rovelli F, Ardizzoia A, Conti A and Maestroni GJM (1993b) A study of the mechanisms involved in the immunostimulatory action of the pineal hormone in cancer patients. *Oncology* **50:**399-402

Masson-Pevet M, Gauer F, Schuster C, Guerreo HY (2000) Photic regulation of mt(1) melatonin receptors and 2-iodomelatonin binding in the rat and Siberian hamster. *Biol Signals Recept* **9**: 188-196.

McArthur AJ, Gillette MU and Prosser RA (1991) Melatonin directly resets the rat suprachiasmatic circadian clock in vitro. *Brain Res* **565:** 158-161

McArthur AJ, Hunt AE and Gillette MU (1997) Melatonin action and signal transductionin the rat suprachiasmatic circadian clock: activation of protein kinase C at dusk and dawn. *Endocrinology* **138:**627-634

Neri B, Brocchi A, Carossino AM, Cini-Neri G, Tommasi MS and Cagnoni M (1995) Effect of melatonin administration on cytokine production in patients with advanced solid tumors. *Oncol Rep* **2:**45-47

Ohdo S, Arata N, Furukubo T, Yukawa E, Higuchi S, Nakano S and Ogawa N (1998) Chronopharmacology of granulocyte colony-stimulating factor in mice. *J*

Pharmacol Exp Ther **285:**242-246

Ohdo S, Grass GM and Lee VHL (1991) Improving the ocular to systemic ratio of topical timolol by varying the dosing time. *Invest Ophthalmol Vis Sci* **32:**2790-2798

Ohdo S, Koyanagi S, Suyama H, Higuchi S and Aramaki H (2001) Changing the dosing schedule minimizes the disruptive effects of interferon on clock function. *Nature Medicine* **7:**356-360

Ohdo S, Ogawa N, Nakano S and Higuchi S (1996) Influence of feeding schedule on the chronopharmacological aspect of sodium valproate in mice. *J Pharmacol Exp Ther* **278:**74-81

Panzer A and Viljoen M (1997) The validity of melatonin as an oncostatic agent. *J Pineal Res* **22:**184-202

Reppert SM and Weaver DR (1995) Melatonin madness. Cell **83:**1059-1062

Shiu SY, Li L, Xu JN, Pang CS, Wong JT and Pang SF (1999) Melatonin-induced inhibition of proliferation and G1/S cell cycle transition delay of human choriocarcinoma JAr cells: possible involvement of MT2 (MEL1B) receptor. *J Pineal Res* **27:**183-192

Starkey SJ (1996) Melatonin and 5-hydroxytryptamine phase-advance the rat circadian clock by activation of nitric oxide synthesis. *Neurosci Lett* **211:**199-202

Starkey SJ, Walker MP, Beresford JM and Hagan RM (1995) Modulation of the rat suprachiasmatic circadian clock by melatonin in vitro. *Neuroreport* **6:** 1947-1951

Sze SF, Ng TB and Liu WK (1993) Antiproliferative effect of pineal indoles on cultured tumor cell lines. *J Pineal Res* **14:**27-33

Takane H, Ohdo S, Yamada T, Yukawa E and Higuchi S (2000) Chronopharmacology of antitumor effect induced by interferon-β in tumor-bearing mice. *J Pharmacol Exp Ther* **294:**746-752

Tenn C and Niles LP (1993) Physiological regulation of melatonin receptors in rat suprachiasmatic nuclei: diurnal rhythmicity and effects of stress. *Molecular and Cellular Endocrinol* **98:**43-48

Vacas MI and Cardinali DP (1979) Diurnal changes in melatonin binding sites of hamster and rat brains. Correlation with neuroendocrine responsiveness to melatonin. *Neurosci Lett* **15:**259-263

Watanabe H, Ohdo S, Ishikawa M and Ogawa N (1992) Effects of social isolation on

pentobarbital activity in mice: Relationship to racemate levels and enantiomers levels in brain. *J Pharmacol Exp Ther* **263:**1036-1045

Footnotes

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Figure legends

Fig.1 (A) Influence of dosing time on tumor weight on day 8 after initiation of daily MLT (1mg/kg,ip) treatment at 07:00, 13:00, 19:00 or 01:00. (B) Influence of daily LZD (10 mg/kg,ip) dosing on the antitumor effect of daily MLT treatment at 13:00 or 01:00. Each point represents the mean with S.E. of 9-10 mice. * $P < 0.05$; compared with 01:00 group using Tukey's test. # $P < 0.05$; ## $P < 0.01$; compared with MLT group using Tukey's test. □, saline; ■, MLT; ▨, MLT+LZD.

Fig.2 Time-dependent change in specific binding of MLT to membrane of tumor cells. The membranes were incubated with [^{3}H]MLT (50.0 nM) at 4 °C for 5 hr. Non-specific binding was determined in the presence of 100 μM MLT. Specific binding is defined as total binding minus non-specific binding. Each point represents the mean with S.E. of 6 mice. * $P < 0.05$; ** $P < 0.01$; compared with 01:00 group using Tukey's test.

Fig.3 The locomotor activity in mice under daily saline (A), MLT (1mg/kg,ip) (B) or MLT and LZD (10 mg/kg,ip) (C) treatment for 7 days at 13:00 (○) or 01:00 (●). The activity count (number of movements) of 3 mice was calculated using a moving average with a 4-hr window after recorded at 1 hr intervals. The average counts were plotted at 09:00, 13:00, 17:00, 21:00, 01:00 or 05:00. * $P < 0.05$; compared with 01:00 group using Student's t-test.

Fig.4 Time-dependent change in specific binding of MLT to membranes of SCN cells prepared at 13:00 (○) or 01:00 (●). (A) Concentration dependence of [^{3}H]MLT binding to membranes of SCN cells. The membranes were incubated with [^{3}H]MLT (12.5-50.0 nM) at 4 °C for 5 hr. Non-specific binding was determined in the presence of 100 μM MLT. Specific binding is defined as total binding minus non-specific binding. Each value is the mean with S.E. of 3 observations. (B) Transformation of the saturation data using the Scatchard method. To fit the data was carried out by the method of least squares. Each value is the mean of triplicate. * $P < 0.05$; ** $P < 0.01$; compared with 01:00 group using Student's t-test.

Fig.5 Influence of dosing time on MLT concentration in tumor after MLT (1mg/kg,ip) injection. Tumor was removed at 30 min after MLT injection at 07:00, 13:00, 19:00 or 01:00. MLT concentration was measured with HPLC method. Each value is the mean with S.E. of 6 observations. There was no statistical significant of difference by ANOVA.

Fig.6 Influence of dosing time on MLT concentration in brain after MLT (1mg/kg,ip) injection. Brain was removed at 30 min after MLT injection at 13:00 or 01:00. MLT concentration was measured with HPLC method. Each value is the mean with S.E. of 6 observations.

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TABLE 1

Time-dependent change in specific binding of MLT to SCN cells prepared at 13:00 or 01:00. Bmax and Kd were calculated using 5 different concentration of ligand with the method of Scatchard (Fig.4). To fit the data was carried out by the method of least squares. Each value is the mean with S.E. of 3 observations.

	Time of SCN preparation (clock hours)		Percentage change
	13:00	01:00	(13:00 / 01:00)
Bmax (fmol/mg protein)	112.1 ± 6.2 *	80.1 ± 7.3	40
Kd (nM)	142.0 ± 8.7	123.7 ± 11.4	15

* P<0.05, compared with the 01:00 group using Student's t-test .

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TABLE 2

Influence of dosing time on plasma MLT concentration after MLT (1mg/kg,ip) injection. Blood samples were drawn by orbital sinus collection using micropipettes at 10, 30, 60 or 120 min after MLT injection at 07:00, 13:00, 19:00 or 01:00. Plasma MLT concentration was measured with HPLC method. Each value is the mean with S.E. of 6 observations.

Time after MLT injection	Time of MLT injection (clock hours)			
	07:00	13:00	19:00	01:00
min	ng/ml			
10	238.5±12.5	190.0±7.5	209.9±11.4	221.4±18.5
30	114.1±3.6	106.6±5.0*	100.9±3.3*	119.2±2.8
60	51.5±3.8	45.9±2.2	45.2±2.5	52.9±3.1
120	5.3±0.9	5.3±0.9	5.5±0.7	5.0±0.6

* $P<0.05$, compared with the 01:00 group using Tukey's test.

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TABLE 3

Influence of dosing time on pharmacokinetic parameters after MLT (1 mg/kg,ip) injection at 07:00, 13:00, 19:00 or 01:00. Each value is the mean with S.E. of 6 observations.

Pharmacokinetic parameters	Time of MLT injection (clock hours)			
	07:00	13:00	19:00	01:00
AUC (ng•hr/ml)	180.4 ± 5.6	153.7 ± 4.9*	157.5. ± 3.6*	178.5 ± 7.2
MRT (hr)	0.47 ± 0.02	0.49 ± 0.01	0.48 ± 0.02	0.48 ± 0.02
CL (ml/min/kg)	92.8 ± 2.7	109.0 ± 3.3*	106.1 ± 2.4*	94.2 ± 3.9
Vd (L/kg)	2.61 ± 0.14	3.22 ± 0.07*	3.08 ± 0.17*	2.70 ± 0.16
Ke (/hr)	2.10 ± 0.10	2.05 ± 0.07	2.01 ± 0.08	2.14 ± 0.09

* P<0.05, compared with the 01:00 group using Tukey's test.

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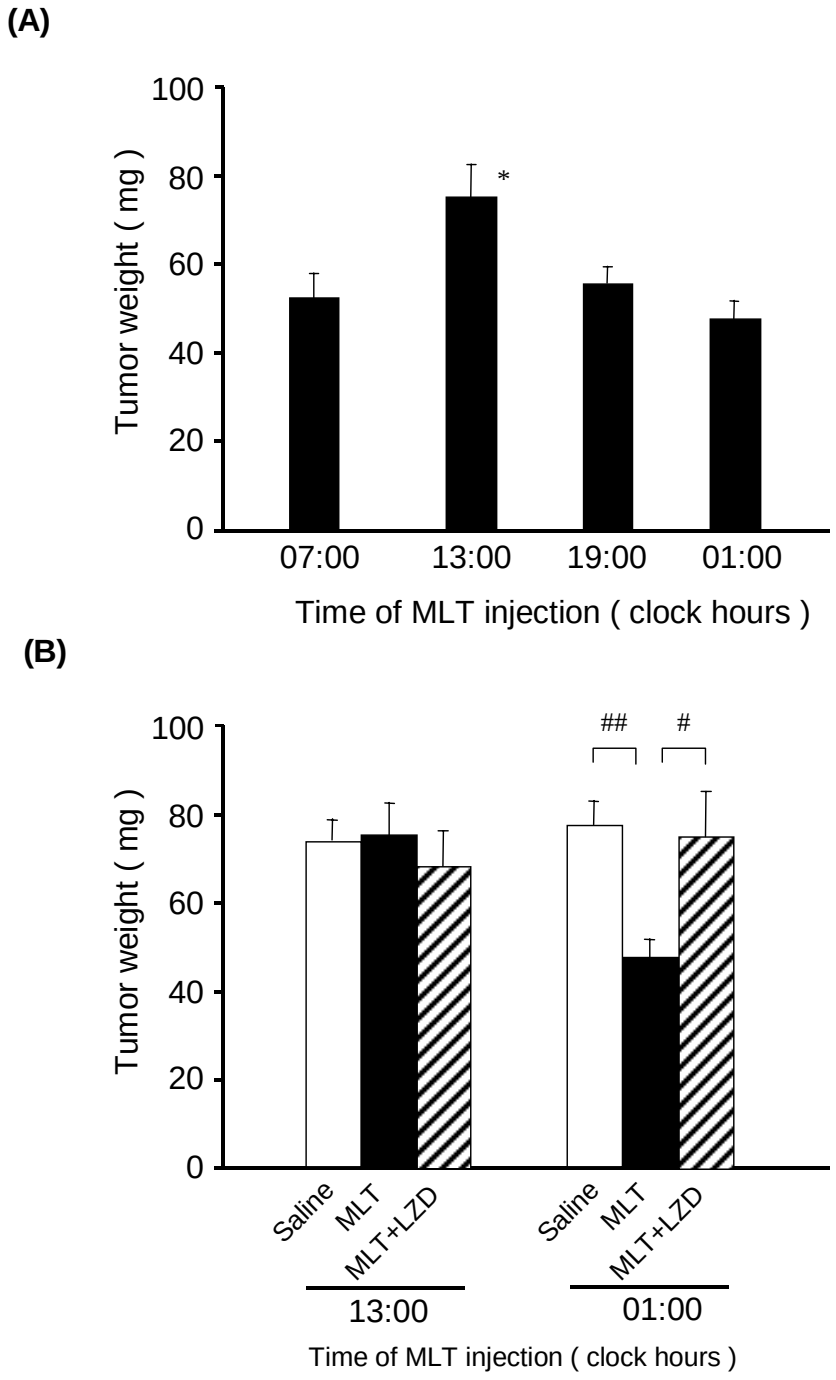

Fig. 1

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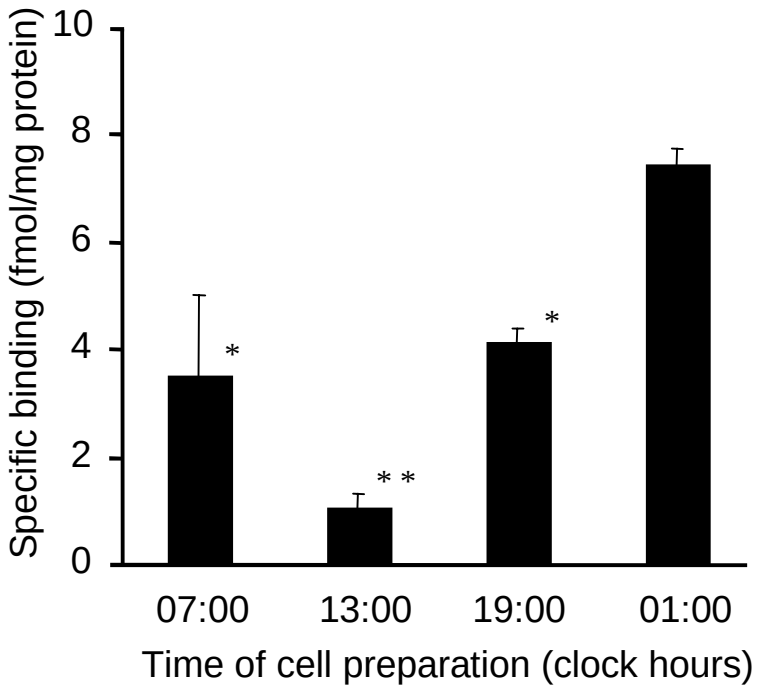

Fig. 2

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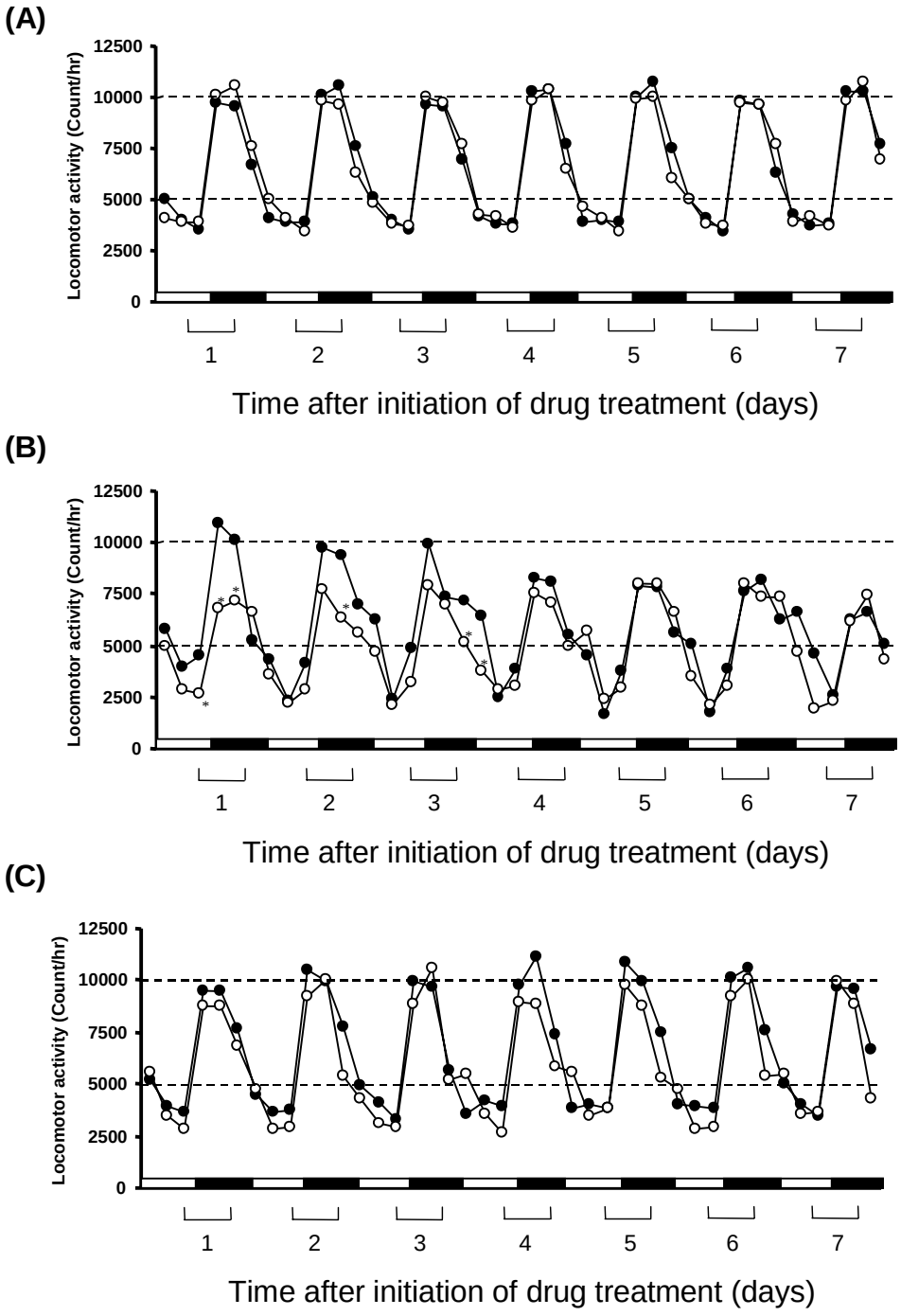

Fig. 3

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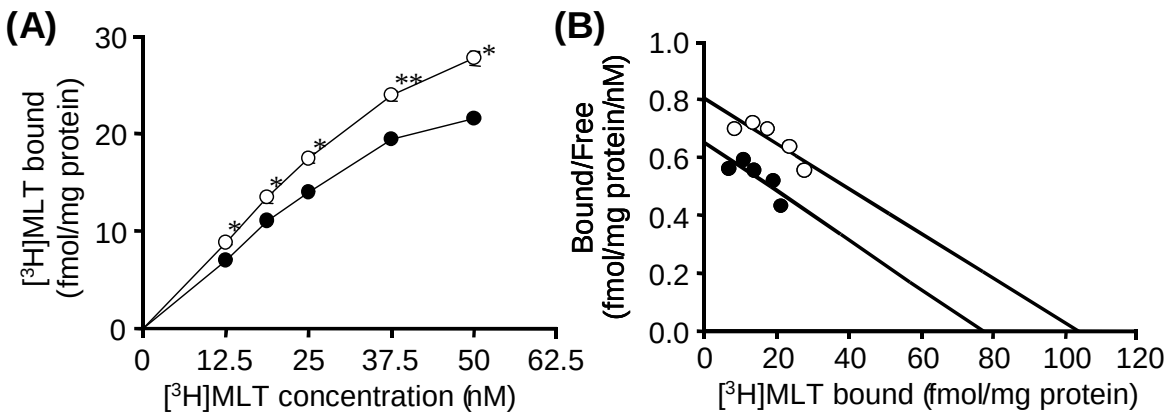

Fig. 4

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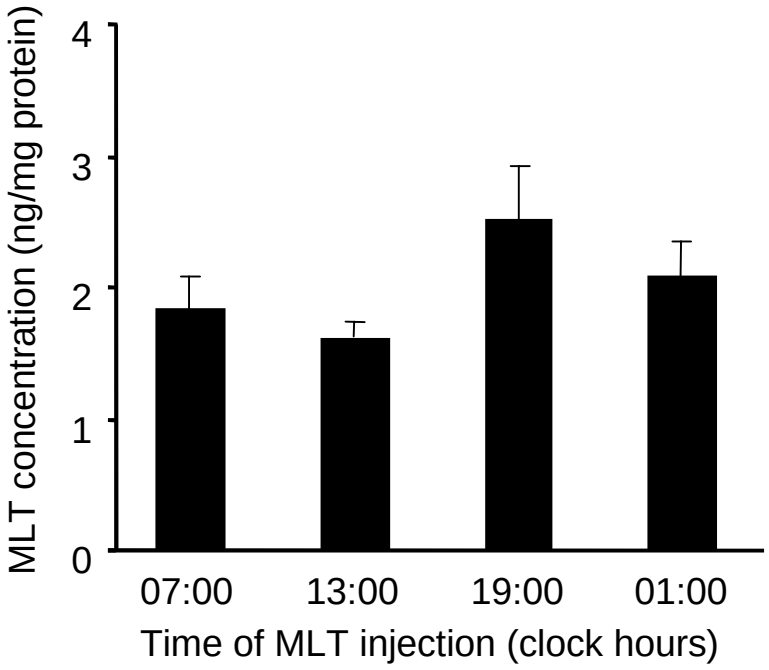

Fig. 5

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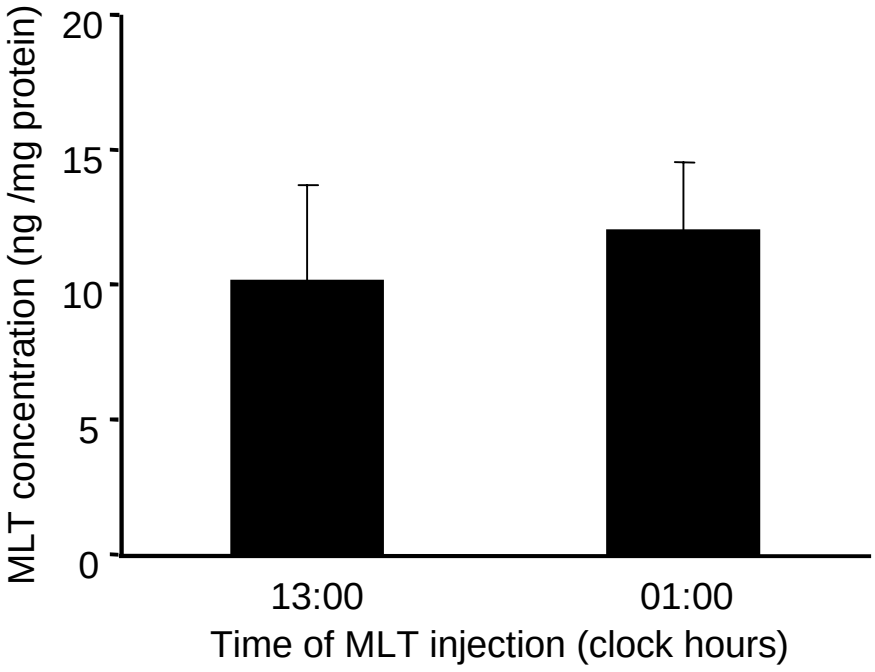

Fig. 6