Melatonin Protects on Toxicity by Acetaminophen But Not on Pharmacological Effects in Mice

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The pineal gland and its main hormone, melatonin (MLT), are involved in a variety of physiological processes. MLT is a member of the indolamine family and has significant antioxidative activity. Acetaminophen (AA) is the most widely used medication in the world, both by prescription and over the counter. In large doses, AA is hepatotoxic causing oxidative stress and lipid peroxidation. Therefore, antioxidants have been used to protect against the toxicity of AA. Here, we examined *in vitro* and *in vivo* the protective effects of MLT against AA-induced toxicity in mice. MLT (100 μM) had a significant protective effect on the AA (7 mM)-induced loss of cell viability in mouse primary cultured hepatocytes as determined using the ^{3}H-thymidine incorporation assay and MTT assay. The AA-induced generation of reactive oxygen species (ROS) peaked at 6 h and was followed by an increase in lipid peroxidation at 12 h in hepatocytes. MLT (0.1, 1, 10 or 100 μM) dose-dependently attenuated the increase in both production of ROS and lipid peroxidation by AA. Similarly, *in vivo*, AA (400, 600 or 800 mg/kg, intraperitonealy)-induced mortality and hepatotoxicity were significantly decreased by MLT (10 mg/kg, subcutaneously). Pretreatment with MLT had a greater protective effect on the hepatotoxicity of AA than post-treatment. However, MLT had no protective effect on the antipyretic effect or antinociception caused by AA. These results suggest that MLT is potentially useful for preventing AA-induced toxicity, but not the antipyretic effect or antinociception caused by AA.

Key words melatonin; acetaminophen; antioxidant; hepatotoxicity

The pineal gland and its main hormone, melatonin (MLT), are involved in a variety of physiological processes including the regulation of endocrine rhythms,[1] antigonadotropic effects,[2] neuroprotective effects,[3] and stimulation of the immune function.[4] In addition, recent studies *in vitro* have shown that MLT functions as an antioxidant, *i.e.* a scavenger of the hydroxy radical and peroxy radical.[5,6] It has also been shown that when animals and tissues are subjected to lipid peroxidation, MLT affords substantial protection against the oxidative destruction of lipids.[7,8]

Acetaminophen (AA) is the most widely used medication in the world, both by prescription and over the counter. In large doses, however, AA produces centrilobular hepatic necrosis in humans and experimental animals.[9,10] The AA-induced hepatotoxicity involves a change in cellular redox status toward a state of oxidative stress.[11,12] Whereas depletion of intracellular gluthathione (GSH) by the active AA metabolite, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), is an early event leading to oxidative stress, reactive oxygen and nitrogen species generated by hepatic nonparenchymal cells and infiltrating phagocytes contribute to the persistence of this response.[13,14] The concept that oxidative stress is important in the pathogenic process is supported by findings that antioxidants abrogate AA-induced hepatotoxicity.[15,16]

Since AA is included in cold remedies as an analgestic or antipyretic and frequently used long-term, there are numerous opportunities for its concomitant use with other drugs. Although not licensed as a drug, MLT is widely sold as a nutritional supplement in many countries for its purported sleep-promoting and antiageing properties.[17] Therefore, it is important to evaluate the interaction between AA and MLT. Recently, Sener *et al.* has shown to protect by MLT on AA-induced hepatotoxicity in mice.[18] The aim of this study was to examine the effects of MLT on AA-induced hepatotoxicity *in vitro* and *in vivo* or pharmacological action by AA using mice.

MATERIALS AND METHODS

Animals and Chemicals Adult male C57BL/6 mice (25—30 g) obtained from Japan SLC (Hamamatsu, Japan) were maintained on a 12 h light/dark cycle in a temperature- and humidity-controlled room. The experiments were conducted in accordance with the standards established by the Japanese Pharmacological Society. The animals were allowed free access to laboratory pellet chow (CE-2; CLEA Japan Inc., Tokyo, Japan) and water before the experiments. AA was purchased from Junsei Pharmaceutical Co., Ltd. (Nagano, Japan). Melatonin (MLT), *N*-acetyl-L-cysteine (NAC), and other drugs of the highest grade available were purchased from Wako Pure Chemical Industries (Osaka, Japan). All cell culture reagents were obtained from Invitrogen Corp. (Carlsbad, CA, U.S.A.). For the experiments *in vitro*, AA was dissolved in medium, and MLT was dissolved in dimethylsulfoxide (DMSO) to make a concentration of 1 M as a stock solution. It was used after dilution of the stock solution with DMSO. DMSO at concentrations lower than 0.25% had no effect on cell growth. For the experiments *in vivo*, AA was dissolved in 0.5% Tween 80 in saline. MLT was initially dissolved in DMSO and diluted with saline to produce a 0.25% DMSO solution. Exposure to light was kept to a minimum for all drugs used.

Hepatocyte Culture Hepatocytes were isolated from the mice by a modified version of the two-step collagenase per-

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fusion method of Seglen.[19] Briefly, the liver of a mouse was perfused for 5 min with prewarmed (37 °C) Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution, pH 7.2, containing 10 mM HEPES, 0.5 mM EGTA, and 4.0 mM $NaHCO_3$. This was followed by a 15 min perfusion with prewarmed (37 °C) Hank's balanced salt solution, pH 7.5, containing collagenase (0.05%) and buffered with 10 mM HEPES and 4.0 mM $NaHCO_3$. After the second step of perfusion, isolated cells were centrifuged ($50\times g$ for 1 min, 4 spins) in minimum essential medium (MEM) to remove nonparenchymatous and dead cells. Then the medium was changed to Williams' E medium (WE) containing 5% fetal bovine serum, 10^{-7} M insulin, 10^{-7} M dexamethasone, 100 units/ml penicillin and 100 μg/ml streptomycin. This procedure routinely yielded over 90% viability based on the trypan blue exclusion test. Approximately 1×10^6/ml parenchymatous cells were plated on 35-mm Falcon collagen-coated type I dishes. After incubation at 37 °C for 2 h in a humidified environment of 5% CO_2–95% air, the cultures were rinsed with warmed phosphate-buffered saline (PBS) to remove free cells and debris, and then serum-free WE containing 0.12 μg/ml aprotinin was added and the cells were incubated with AA and MLT or NAC.

Cell Viability Cell viability was evaluated by measuring the level of ^{3}H-thymidine incorporation or using a modification of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay.[20] Briefly, ^{3}H-thymidine at a final concentration of 0.05 mCi/ml was added to cultured hepatocytes 30 min before the measurement time points. The cultured hepatocytes were washed with PBS two times, and then the cells were harvested from the dishes with a cell scraper. Samples were emulsified in scintillation fluid and measured in a Beckman LS 6000TA beta scintillation counter. ^{3}H-thymidine incorporation (%) was calculated according to {cpm (test groups)/cpm (control groups)}$\times$100. For MTT assay, following exposure of hepatocytes to AA and MLT or NAC, 50 μl of MTT (10 mg/ml saline) was added, and samples were incubated for 1 h at 37 °C. The cells were lysed and solubilized by addition of 500 μl of dimethyl sulfoxide (DMSO) for 2 h at 37 °C, and the absorbance of 100-μl aliquots was determined at 590 nm using an Inter-med model NJ-2300 Microplate Reader. Cell viability (%) was calculated relative to the control.

Generation of Reactive Oxygen Species (ROS) The generation of intracellular reactive oxygen species (ROS) was evaluated based on the intracellular peroxide-dependent oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to form the fluorescent compound, 2′,7′-dichlorofluorescein (DCF), as previously described by us.[21] After the treatment with drugs, the cells were incubated with 20 μM DCFH-DA (from a stock solution of 4 mM DCFH-DA in ethanol) at 37 °C for 10 min. The culture medium was removed, the cells were washed with PBS, and 2 ml of PBS was added to each well. The fluorescence intensity of the cell suspension was determined using a fluorescence spectrophotometer (RF-1500, Shimadzu, Kyoto, Japan) with excitation at 488 nm and emission at 525 nm. The untreated groups were used as the control. The results are expressed as a percentage of the fluorescence intensity with respect to the control.

Lipid Peroxidation The lipid peroxide level was assayed by fluorimetrically measuring the level of malondialdehyde (MDA) in hepatocytes using the method of Yagi.[22] Cells were exposed to drugs, and then washed with PBS, scraped and homogenized in ice-cold 1.15% KCl. Samples containing 20 μl of cell lysate were combined with 20 μl of 8.1% sodium dodecyl sulfate (SDS), 150 μl of 20% acetic acid adjusted to pH 3.5, and 150 μl of 0.8% thiobarbituric acid (TBA). The mixture was heated at 95 °C for 60 min. After cooling to room temperature, 100 μl of distilled water and 2.0 ml of a mixture of *n*-butanol and pyridine (15 : 1, v/v) were added to each sample and the mixture shaken was vigorously. After centrifugation at 1200 rpm for 10 min, the supernatant was isolated. The fluorescence intensity was measured using the RF-1500 (Shimadzu) with excitation at 515 nm and emission at 553 nm. A MDA solution made freshly by the hydrolysis of 1,1,3,3-tetraethoxypropane (TEP) was used as the standard. The results are expressed as nmol MDA/mg protein.

Hepatotoxicity and Mortality *in Vivo* The Effect of MLT on the hepatotoxicity of AA *in vivo* was estimated by measuring levels of serum glutamyl oxaloacetic transaminase (GOT) and glutamyl pyruvic transaminase (GPT) activity. These activities were monitored with a commercial kit from Wako Pure Chemical Industries (Osaka, Japan). Acute mortality was recorded for 72 h after the intraperitoneal (i.p.) injection of AA.

Temperature Measurement A rectal probe for mice and a thermocouple-monitoring thermometer were obtained from Natume Industries (Tokyo, Japan). The probe was inserted into the rectum to a depth of 2 cm. All experiments began with the i.p. administration of AA at 10 am, and temperature measurements were made 1 h thereafter.

Nociceptive Assessment Mice were assessed for nociceptive sensitivity using the writhing test as described by Koster *et al.*[23] All mice were acclimatized to the procedure room for at least 1 h prior to testing. All testing occurred near midphotoperiod (10:00—16:00). Mice were placed on a glass surface within transparent Plexiglas cylinders and allowed 30 min to habituate to the cylinder. At this point, the mice were weighed, injected with drugs or vehicle, and then placed back into the cylinder. Thirty minutes later, 0.9% acetic acid in saline was injected i.p. (10 ml/kg). Mice were placed in the cylinders once again and observed continuously for 30 min. Stereotypical writhes (lengthwise constrictions of the torso with a concomitant concave arching of the back) were counted over this period in 5-min bins. Four mice were observed and scored at a time. In all cases, the experimenter was kept blind to the drug/vehicle solutions. Antinociception was quantified by reference to the vehicle-treated control group.

Statistical Analysis Statistical analysis of the results was performed with a one-way analysis of variance (ANOVA) followed by Scheffe's *F* test. A *p*-value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Cell Viability First, we examined the effect of melatonin (MLT) on the acetaminophen (AA)-induced loss of cell viability in mouse primary cultured hepatocytes (Fig. 1A). MLT (100 μM) and AA (7 mM) were administered simultaneously

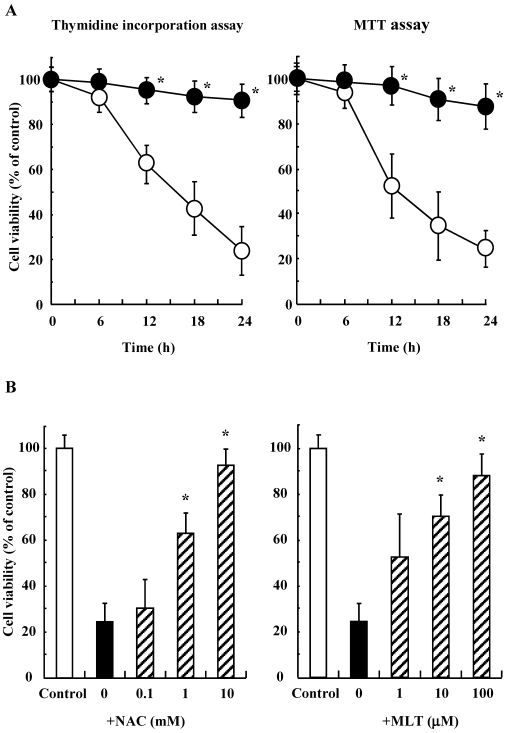

Fig. 1. The Effect of Melatonin (MLT) on the Acetaminophen (AA)-Induced Loss of Cell Viability in Mouse Primary Cultured Hepatocytes

(A) Time-dependent effect of MLT (100 μM) on AA (7 mM)-induced reduction of cell viability. Open circles: AA treatment groups. Closed circles: MLT plus AA treatment groups. (B) Dose-dependent effect of *N*-acetyl-L-cysteine (NAC) and MLT on AA (7 mM)-induced loss of cell viability after treatment for 24 h. The reduction in cell viability was estimated using the ^{3}H-thymidine incorporation assay and MTT assay, as described in Materials and Methods. Each value represents the mean±S.E. for three different experiments performed in triplicate. $*p<0.05$ *vs.* AA (7 mM) treatment groups.

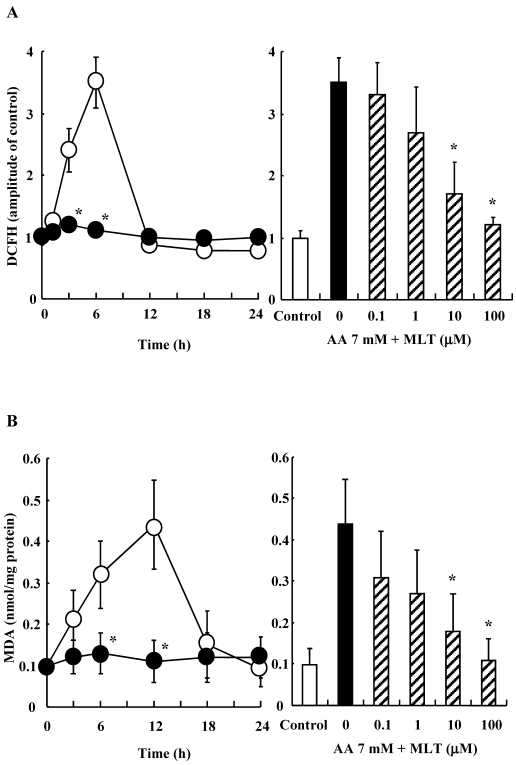

Fig. 2. The Effect of Melatonin (MLT) on Acetaminophen (AA)-Induced Generation of Reactive Oxygen Species (ROS) and Increase in Lipid Peroxidation in Mouse Primary Cultured Hepatocytes

(A) Left panel: Time-dependent effect of MLT (100 μM) on AA (7 mM)-induced generation of ROS. Open circles: AA treatment groups. Closed circles: MLT plus AA treatment groups. Right panel: Dose-dependent effect of MLT on AA -induced generation of ROS for 6 h. The generation of ROS was evaluated using DCFH fluorescence, as described in Materials and Methods. (B) Left panel: Time-dependent effect of MLT (100 μM) on AA (7 mM)-induced increase in lipid peroxidation. Open circles: AA treatment groups. Closed circles: MLT plus AA treatment groups. Right panel: Dose-dependent effect of MLT on AA-induced increase in lipid peroxidation for 12 h. The lipid peroxidation level was evaluated by fluorimetrically measuring the level of malondialdehyde (MDA), as described in Materials and Methods. Each value represents the mean±S.E. for three different experiments performed in triplicate. $*p<0.05$ *vs.* AA (7 mM) treatment groups.

to the hepatocytes. The concentration of AA (7 mM) used in the experiments was reported to reduce cell viability in our previous study.[21] As shown in Fig. 1A, MLT had a significant protective effect on cell viability from 12 h in the ^{3}H-thymidine incorporation assay and MTT assay. MLT alone at more than 5 mM caused a reduction in viability (data not shown). We also examined the dose-dependent effect of MLT on the AA-induced loss of cell viability in the hepatocytes (Fig. 1B). A typical antioxidant, *N*-acetyl-L-cysteine (NAC) is a cysteine precursor known to scavenge free radicals and replenish tissue glutathione (GSH) levels. Treatment of humans and animals with NAC ameliorates the hepatotoxicity of AA.[24,25] Based on these reports, we used NAC as a positive control in this experiment. The AA-induced decline in cell viability was dose-dependently reduced by NAC and MLT. Interestingly, MLT had a protective effect at a lower dose than NAC. MLT is lipophil product than NAC. It is possible to effect of cellular uptake of NAC and MLT depend on their difference of lipophilia. MLT was reported to stimulate the activities of several antioxidant enzymes including superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in hepatocytes.[26] Transgenic mice over-expressing SOD or GSH-Px are protected from the hepatotoxicity of AA.[27] This may be due to the greater efficacy of MLT in scavenging free radicals and also its ability to stimulate antioxidant enzymes, which is not observed with NAC.

Generation of Reactive Oxygen Species (ROS) and Lipid Peroxidation A significant amount of evidence points to the potential involvement of oxidative stress in the toxicity of AA. We attempted to confirm the effect of MLT (0.1, 1, 10 or 100 μM) on the AA (7 mM)-induced generation of reactive oxygen species (ROS) and lipid peroxidation (Figs. 2A, B). The AA-induced generation of ROS peaked at 6 h (Fig. 2A), followed by a maximal increase in lipid peroxidation at 12 h (Fig. 2B). These results suggest that after exposure to AA, hepatocytes undergo a sequence of events, an increase in the generation of ROS, followed by lipid peroxidation, and ultimately a loss of cell viability. MLT dose-dependently attenuated the increase in both the generation of ROS and lipid peroxidation by AA. When the co-incubation with 100 μM MLT, it reduced the ROS and lipid peroxidation until near the levels of control. Cuzzocrea and Reiter suggested that MLT is a better antioxidant against lipid peroxidation than vitamins.[6] Thus MLT has a potential antioxidative effect on the AA-induced generation of reactive oxygen species (ROS) and lipid peroxidation.

Mortality and Hepatotoxicity *in Vivo* As shown in Figs. 1 and 2, the AA-induced loss of cell viability, generation of ROS, and increase in lipid peroxidation were significantly abolished by MLT in mouse primary cultured hepatocytes. Next, we examined *in vivo* the effect of MLT

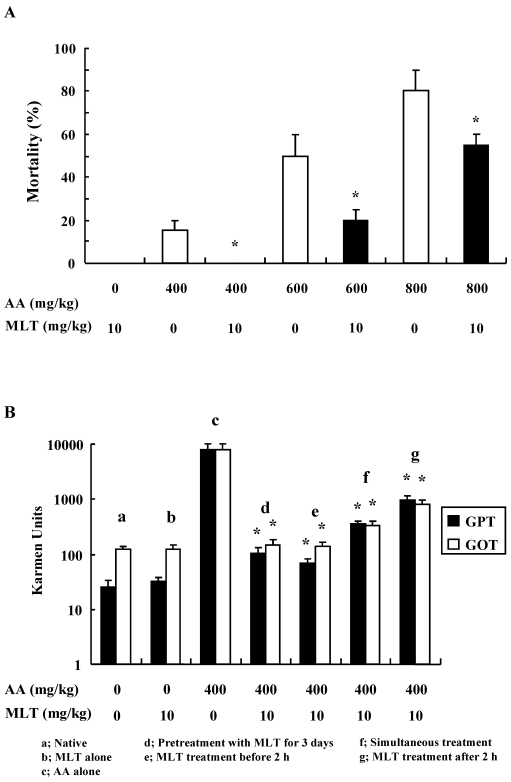

Fig. 3. The Effect of Melatonin (MLT) on Acetaminophen (AA)-Induced Hepatotoxicity and Mortality in Mice

(A) The effect of MLT on mortality in AA-treated mice. Acute mortality was recorded over the 72 h after the intraperitoneal injection of AA. (B) The effect of MLT on the hepatotoxicity of AA in mice. Hepatotoxicity was estimated by measuring levels of serum glutamyl oxaloacetic transaminase (GOT) and glutamyl pyruvic transaminase (GPT) activity for 18 h, as described in Materials and Methods. Each value represents the mean±S.E. for three different experiments (n=10). $*p<0.05$ *vs.* AA alone treatment groups.

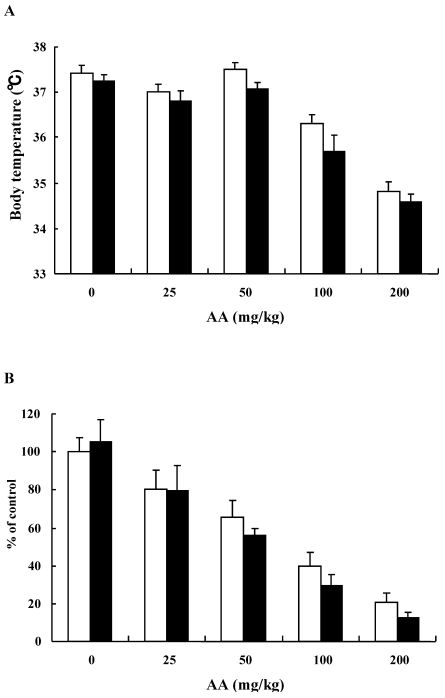

Fig. 4. The Effect of Melatonin (MLT) on Acetaminophen (AA)-Induced Antipyretic Effect (A) and Antinociception (B) in Mice

Open column: AA treatment groups. Closed column: MLT (10 mg/kg, s.c.) plus AA treatment groups. The experimental procedures were described in Materials and Methods. Each value represents the mean±S.E. for five different experiments (n=4).

(10 mg/kg, subcutaneously; s.c.) on mortality and hepatotoxicity in AA-treated mice (Figs. 3A, B). MLT and AA were administered simultaneously. The mortality for AA at 400, 600, or 800 mg/kg (i.p.) alone (n=10) was 15±5, 50±10, and 80±10%, respectively (Fig. 3A). A single treatment with MLT (10 mg/kg, s.c.) has never shown the mortality. Co-treatment with MLT significantly reduced the mortality (AA 400 mg/kg, 0%; AA 600 mg/kg, 20±5%; AA 800 mg/kg, 55±5%). The hepatotoxicity of AA was estimated by measuring transaminase activity, such as GPT and GOT, after treatment for 18 h (Fig. 3B). This was the time point at which the increase in transaminase activity caused by AA peaked in our previous report.[28] AA (400 mg/kg, i.p.) alone remarkably increased levels of GPT (8125±1923 KU/l) and GOT (8028±1743 KU/l) activity compared to the control group (33.8±4.8 and 108±8.8 KU/l, respectively). MLT (10 mg/kg, s.c.) alone treatment group has no change the control group (GPT, 35.6±5.2 KU/l; GOT, 107±11.8 KU/l). We examined the timing of the effect of MLT on the hepatotoxicity of AA. Pretreatment for 3 d, or treatment 2 h before, simultaneously, or 2 h after all significantly ameliorated the AA-induced rise in GPT and GOT activity, 102.8±24.8 and 142.7±37.6 KU/l, 82.5±13.8 and 136.7±29.4 KU/l, 345.4±55.8 and 336.5±63.4 KU/l, and 944.6±210.1 and 882.6±145.3 KU/l, respectively. Pretreatment had a greater effect on the hepatotoxicity of AA than post-treatment. The results suggest that MLT prevents the hepatotoxicity and which does not mean direct action to toxicity caused by AA. The depletion of liver tissue GSH was restored by MLT and/or NAC treatment.[29] Grewal and Racz reported that late treatment with NAC had no effect on the toxicity of AA,[30] however, NAC is used for detoxification in a variety of diseased states including AA toxicity. Pierrefiche *et al.* suggested that the hepatic metabolite of MLT, 6-hydroxymelatonin sulfate, may also be a free radical scavenger since it was shown to be capable of resisting lipid peroxidation.[31] These results suggest that MLT may act not only to prevent AA toxicity but also as a detoxification medicine.

Pharmacological Effect In general, it is thought that the pharmacological effect is weakened if the toxicity is attenuated. We also examined the effect of MLT on the pharmacological action of AA as an antipyretic effect and antinociception (Figs. 4A, B). MLT and AA were administered simultaneously. Body temperature in the non-treatment (control) group and MLT (10 mg/kg, s.c.) treatment group was 37.41±0.19 and 37.23±0.14 °C, respectively. AA at 25, 50, 100, or 200 mg/kg (i.p.) caused hypothermia, which increased dose-dependently at 36.77±0.18, 37.51±0.14, 36.32±0.19, and 34.82±0.19 °C, respectively (Fig. 4A). MLT slightly increased the antipyretic effect of AA (AA 25 mg/kg, 36.81±0.22 °C; AA 50 mg/kg, 37.06±0.14 °C; AA 100 mg/kg, 35.69±0.16 °C; AA 200 mg/kg, 34.45±0.19 °C). Similarly, the antinociceptive effect of AA at 25, 50, 100, or 200 mg/kg (i.p.) dose-dependently increased 80.51±10.12, 65.51±9.18, 39.66±7.14, or 20.34±5.32%. MLT caused a slight increase in AA-induced antinociception (AA 25 mg/kg, 79.32±13.64%; AA 50 mg/kg, 55.63±3.89%; AA 100 mg/kg, 29.41±5.69%; AA 200 mg/kg, 12.44±3.20%). MLT had no protective effect on either the antipyretic effect or antinociception caused by

AA (Figs. 4A, B). There are a number of points at which MLT may interfere with the inflammatory process.[6] Prostaglandin (PG) levels in exudates and cyclooxygenase-2 expression in carrageenan-treated rats were found to be completely suppressed by MLT.[32] There was no significant difference in pharmacological action between the control group and MLT alone treatment group, but MLT may potentiate the effects of other antiinflammatory drugs.

We have used MLT at 10 mg/kg for *in vivo* studies and 100 μM for *in vitro* studies, which dosage too high as compared with the concentration of MLT under physiological conditions. However, half-life of MLT in the blood is relatively short (20—40 min), tissue levels of MLT remain higher than physiological levels when blood MLT concentration fall after administration of a pharmacological dose of MLT.[33] The mechanisms of MLT action in physiological and pharmacological concentration may be different.

In conclusion, our results suggest that MLT has a potentially useful preventive effect on the toxicity of AA, but no effect on the antipyretic effect and antinociception caused by AA.

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