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Studies on the Antiinflammatory, Immunoregulatory, and Analgesic Actions of Melatonin

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Strategy, Management and Health Policy				
Venture Capital Enabling Technology	**Preclinical Research**	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT In order to develop melatonin (MT) as a potential new drug for the treatment of diseases with inflammation, pain, and abnormal immune responses, the effects and mechanisms of MT on inflammation, immunoregulation, and nociception were studied systematically. MT (40–160 mg/kg, ip) had significant analgesic effects in the hot-plate, writhing, and tail-flick models, with a marked dose- and time-dependence. The onset of its analgesia about 30–60 min after ip, was slower than that of pethidine, but the duration was longer (about 1.5–2 h). The analgesia was also induced by icv MT (0.25 mg/kg) injection. A lower dose of MT (10 mg/kg) could enhance the analgesic effect of pethidine, which was blocked by naloxone (10 mg/kg). MT (100 mg/kg, ip) decreased the content of beta-endorphin in the hypothalamus and pituitary. The analgesia of MT could be attenuated by pretreatment with reserpine (30 mg/kg, ip) or phentolamine (10 mg/kg, ip). $CaCl_2$ (230 mg/kg) could antagonize the analgesia of MT. EGTA and verapamil had opposite effects to $CaCl_2$. No tolerance and dependence to MT was found in mice. Further studies showed that MT could enhance the functions of T and B lymphocytes and macrophages in vitro and in adjuvant arthritis, and inhibit the disturbance of immune cells. MT could inhibit the swelling of hindpaw induced by carrageenin and complete Freund's adjuvant. These factors suggest that MT possesses marked antiinflammatory, immunoregulatory, and analgesic effects which may be related to the system of opiate, monoamine, and Ca^{2+} modulation. Drug Dev. Res. 39:167–173.

Key words: melatonin; analgesia; immune; inflammation

INTRODUCTION

Despite the large numbers of studies of analgesia, pain remains one of the most difficult and widespread problems in medicine. Opiates have remained the mainstay of pain management for hundreds and probably thousands of years. However, their use has a price which includes tolerance, physical dependence, respiration and immunity depression. The second potential approach to analgesia is antiinflammatory agents; however, in this case a potent analgesia has not been achieved. A potent nonaddicting analgesia is still needed. Melatonin, N-acetyl-5-methoxytryptamine (MT), is an indoleamine compound mainly produced by the pineal gland following a circadian pattern. Many papers had shown that MT has extensive actions with little toxicity. Early research about the action of MT in the central nervous system (CNS) reported that MT had mild sedative effects, but this was typically considered to be a pharmacological "side-effect" [Barchas et al., 1967]. More recently, the hypnotic effects of MT have been considered an integral part of the physiological role of MT. It was thought that MT is likely to be an effective hypnotic agent for sleep disruption associated with elevated temperature due to low circulating MT levels [Dawson and Encel, 1993]. MT also plays a role in immune regulation [Maestroni, 1993], such as MT stimulation of activated T cells to release immune-opioid peptide. In any case, the fact that immune function is subject to opiatergic modulation via opiate receptors on

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its cellular components has profound biological implications. On the basis of these obervations, we decided to investigate the effects and mechanisms of MT on inflammation, immune responses, and nociception.

MATERIALS AND METHODS

House Hot-Plate

Mice were placed on a heated plate of 55 ± 0.5°C and the reaction time for reflex responses was recorded. The key responses included paw-licking, limb withdrawal, and escape attempts. The mice with latencies less than 5 sec or more than 30 sec were excluded. The latency was examined twice, then the average value was calculated. The latency was limited to 60 sec after MT (Sigma, St. Louis, MO) administration. The analgesic effect (%) was the ratio of the increased latency (sec) after drug administration to the latency (sec) before treatment. The ED_{50} was estimated by probit analysis.

Writhing Assay

The ip injection of acetic acid to mice resulted in peritoneal irritation which elicited a writhing response consisting of abdominal constriction, turning the trunk, and extension of the hind legs. The total numbers of writhing movements within 15 min were recorded under room temperature (20 ± 1°C).

Intracerebroventricular Injection

Mice were fixed stereotaxically and the injection point located at 2 mm anterolateral to the cross of the sagittal fissure and the ear-line. For injection, a needle of a 10 μl syringe was inserted with its tip extended 2.5 mm into the cerebral ventricle. Before injection, the syringe was washed in 70% ethanol. MT (10 μl) was injected within 5 sec, and the syringe remained in for another 10 sec. The analgesia was observed by means of hot-plate. Stain (0.05 ml) was injected with the same procession at the end of the experiment, and the brain was removed to localize the place where the drug reached.

Rat Tail-Flick

The radiant heat source used in the experiment was a complete reflexing light bulb (12 V, 75 W, 7.5 mm). The light was converged with a convex lens of focus 3.5 cm. A small metal ring was placed in front of the lens on purpose to keep the stimulating intensity stable with repeated testing and the stimulating place on the focus point. The distance between the ring and the lens was 3.4 cm and the diameter of the ring was similar to that of the light point. In the testing procedure, the rat tail was placed 1 mm anterior to the ring. The predrug latency was about 5 sec. The base-line reaction time was measured 3 times at 1-min intervals and then the average value was calculated. The latency was measured twice at 30-min intervals after drug administration. The change of latency (%) was the ratio of the increased latency after drug administration to the latency before drug administration.

Mouse Tail-Erecting

The mice were administered sc with MT (350 mg/kg) and morphine (30 mg/kg). Two hours later, the tail-erecting phenomenon was observed.

Naloxone-Induced Jump in Mouse

MT and morphine were administered to mice at the equianalgesic dose twice a day for 5 days. Naloxone (Nal 5 mg/kg) was injected ip 4 h after the last administration of drugs. Then the mouse was placed on a round table of 35 cm height and 35 cm diameter and the jump induced by naloxone was observed for 10 min.

Immune Parameters Assay

The proliferation of lymphocytes was determined using [^{3}H]-TdR incorporation. Interleukin-1 (IL-1) and interleukin-2 (IL-2) were estimated, respectively, using the thymocyte proliferation and conA-activated splenocyte proliferation assay in CF7BL 6J mice [Liang et al., 1989; Wei et al., 1989]. Macrophage function was assessed in accordance with the method of Chen et al. [1991].

Inflammatory Models in Rats

Carrageenin-induced edema hindpaw and adjuvant-induced arthritis of rats were performed according to procedures described by Wei et al. [1986].

beta-EP RIA

The experiment was done according to procedures described by Zhu et al. [1986].

RESULTS

Analgesic Effects and Characteristics of MT

Mice were divided into 7 groups, which were MT (10, 20, 40, 80, 160 mg/kg), pethidine (30 mg/kg), and vehicle groups. The analgesia produced by MT was estimated in hot plate, writhing, and tail-flick assays, respectively. It was found that MT (10–60 mg/kg) had significant analgesic effects, which depended upon dose and time. Potency of MT at the dose of 20 mg/kg was equivalent to pethidine in hot-plate. MT had a slower onset of action than pethidine, the peak effect appearing 1 hr after administration (Figs. 1–3). The ED_{50} value of MT analgesia was 34.78 mg/kg, which was similar to that of pethidine (34.66 mg/kg) estimated by probit analysis in the hot-plate test. The ED_{50} values were 38.85 and 34.07 mg/kg in writhing and tail-flick, respectively. The analgesia was also found by icv MT (0.25, 0.5, 1, 0, 2.0 mg/kg). Figure 4

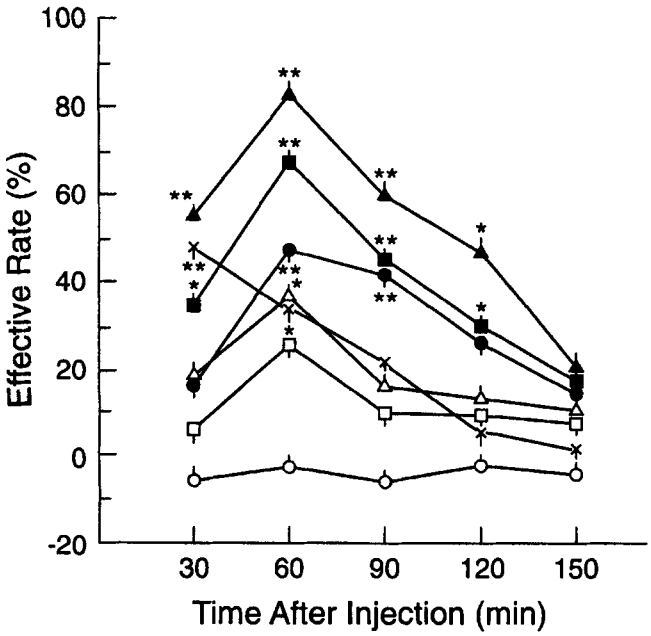

Figure 1. Analgesic effects of MT (ip) and PD (ip) in mice hot-plate. N = 10, Mean (X) ± standard deviation (SD). *$P < 0.05$, **$P < 0.01$, compared with MT vehicle. ○–○, MT vehicle; □–□, MT 10 mg/kg; △–△, MT 20 mg/kg; ●–●, MT 40 mg/kg; ■–■, MT 80 mg/kg; ▲–▲, MT 160 mg/kg; X–X, PD 40 mg/kg, MT vehicle: 10% propylene glycol.

shows the analgesic property of MT and the dose- and time-effect relationship following icv administration. The ED_{50} was 0.38 mg/kg, which was lower than that by ip administration.

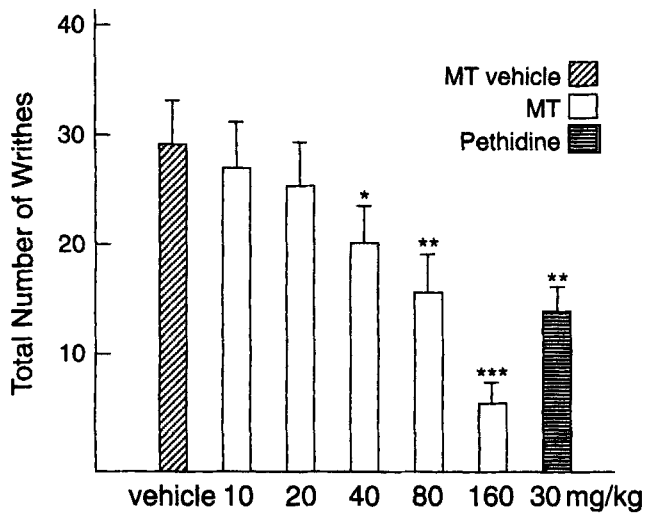

Figure 2. Effects of ip MT and PD on acetic acid-induced writhing response in mice. N = 10, X ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with MT vehicle.

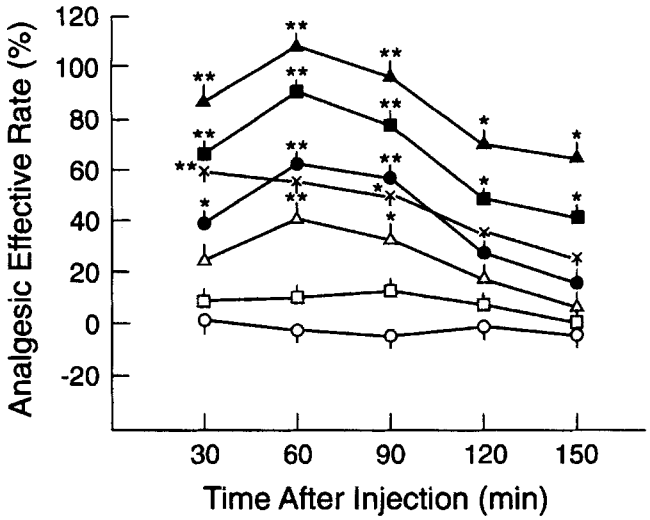

Figure 3. Analgesic effects of MT and PD on mice tail-flick model, MT, and PD were treated ip at 8 AM. N = 10, X ± SD. *$P < 0.05$, **$P < 0.01$ compared with MT vehicle. ○–○, MT vehicle; □–□, MT 10 mg/kg; △–△, MT 20 mg/kg; ●–●, MT 40 mg/kg; ■–■, MT 80 mg/kg; ▲–▲, MT 160 mg/kg; X–X, PD 30 mg/kg.

Addiction Research

Addiction produced with MT was investigated in mice by means of tolerance, tail-erecting, and naloxone-induced jump tests. MT (40 mg/kg ip), administered in 7 days consecutively to mice, did not shorten the latency, but pethidine did (Fig. 5). A large dose of MT (350 mg/kg) was given; no tail-erecting phenomonen was observed, but the tail-erecting response appeared in the morphine group, which was not restored to normal until

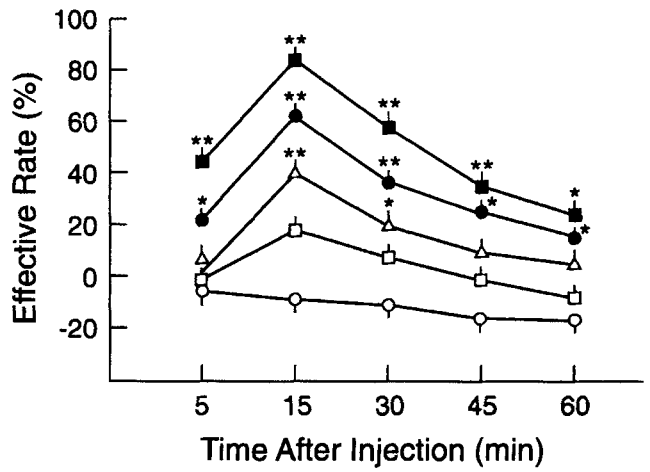

Figure 4. Analgesic effects of MT and PD in mice hot-plate by icv. At 8 AM. N = 10, X ± SD. *$P < 0.05$, **$P < 0.01$, compared with ACSF. ○–○, ACSF; □–□, MT 0.25 mg/kg; △–△, MT 0.5 mg/kg; ●–●, MT 1.0 mg/kg; ■–■, MT 2.0 mg/kg. ACSF: Artificial cerebrospinal fluid.

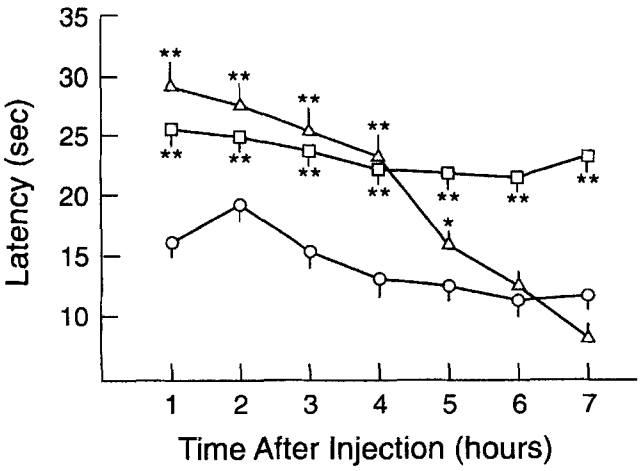

Figure 5. Analgesic effects of MT and PD in mice hot-plate for 7 days. Each drug was ip. At 8 AM. N = 10, X ± SD. *$P < 0.05$, **$P < 0.01$, compared with MT vehicle. O–O, MT vehicle; □–□, MT 40 mg/kg; △–△, PD 40 mg/kg.

2 h passed. Naloxone precipitation showed that MT (70, 700 mg/kg, × 5 days) did not cause jumping of mice, but the mice in the morphine group jumped accompanied with tremor, hyperventilation, and diarrhea (Table 1).

Analgesic Mechanisms of MT

Mice, divided into groups according to Table 2, were injected sc with naloxone (2 or 10 mg/kg) 10 min before MT administration; 40 min later, 0.6% acetic acid (10 mg/kg) was given. Table 2 demonstrates that MT can potentiate the analgesic effect of pethidine, which can be blocked by naloxone. Table 3 shows that the content of beta-EP in the hypothalamus and pituitary decreased significantly 1 h after ip MT (10 mg/kg) in the hot-plate test.

Relationship Between MT Analgesia and Monoamine Transmitters

Monoamine receptor antagonist and monoamine transmitter uptake inhibitors were used in this experiment. Phentolamine (10 mg/kg) and reserpine (3 mg/kg) were given 30 min and 3 h, respectively, before MT injection. The changes of threshold are presented in Figure 6. Reserpine and phentolamine could abolish the analgesia of MT.

TABLE 1. Effect of MT on Naloxone-Induced Jump in Mice

Group	Dose (mg/kg × 5 days)	Naloxone (5 mg/kg, ip; jump rate)	Precipitation (average value of jump)
MT vehicle	—	0/20	0
MT	70	0/20	0
	700	0/20	0
Morphine	60	20/20	3.1

TABLE 2. Effects of PD and Nal on the Analgesic Effect of MT in Mice (X ± SD, N = 10)†

Drug	Dose (mg/kg)	Number of writing
MT vehicle	—	27.4 ± 7.1
MT	10	27.1 ± 6.8
PD	10	26.5 ± 7.0
MT+PD	10+10	20.0 ± 6.6*,**
MT	40	18.2 ± 5.3*
MT+Nal	40+2	20.1 ± 6.4*
MT+Nal	40+10	27.3 ± 6.9***

†PD: pethidine; Nal: naloxone.
*$P < 0.05$, compared with MT vehicle group, **$P < 0.05$, compared with MT (10) or PD (10) group.
***$P < 0.01$, compared with MT (40) group.

Relationship Between MT Analgesia and Ca^{2+} Channel Function

$CaCl_2$, verapamil (Ca^{2+} antagonist), and EGTA (Ca^{2+} chelator) were given 10 min before MT administration. One hour later latency was recorded in the hot-plate test. Table 4 shows that $CaCl_2$ lowered the pain threshold in MT (40 mg/kg) group, while verapamil and EGTA enhanced the analgesia of MT.

Relationship Between MT Analgesia and the Immune Response

ConA-induced proliferation of splenocytes was enhanced and the phagocytosis of macrophages was potentiated after administration of MT of 40 mg/kg (for 7 days) and 700 mg/kg (for 5 days), respectively (Table 5). MT (0.01–10 μmol/L) could enhance ConA- and LPS-induced splenocyte proliferation and the production of IL-1 and IL-2 in vitro (Figs. 7, 8). In addition, MT (10 μg/kg) inhibited the disorder of T lymphocytes and macrophage function in adjuvant arthritis in rats (Table 6).

Antiinflammatory Action of MT

Figure 9 shows that MT (10, 200, or 4,000 μg/kg, ip) significantly attenuates the edema of hindpaw induced by carrageenin. This suggests that MT plays an important suppressive role in the early inflammatory response. Table 7 shows that MT (10 μg/kg, ip) inhibited the secondary edema in adjuvant arthritis in rats. This indicates that MT can inhibit the secondary inflammation.

TABLE 3. Effect of MT (100 mg/kg) on β-EP Content in the Hypothalamus and Pituitary in Rat (X ± SD, N = 6)

Group	Pain threshold	Content of β-EP	
		Hypothalamus (pg/kg)	Pituitary (ng/kg)
MT vehicle	15.4 ± 4.1	84.0 ± 12.7	182.1 ± 8.9
MT	24.2 ± 7.8*	63.9 ± 7.8**	153.3 ± 21.6*

*$P < 0.05$, **$P < 0.01$ compared with MT vehicle.

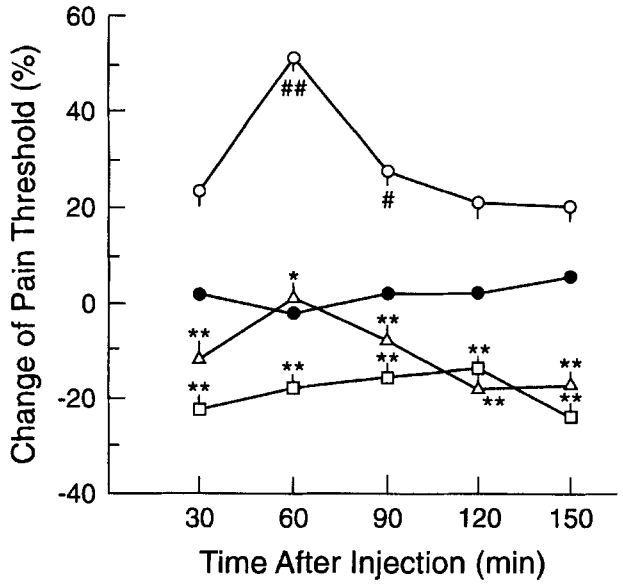

Figure 6. Effects of phentolamine and reserpine on the analgesia of MT in mice hot-plate. N = 10, X ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with MT vehicle; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with MT 40 mg/kg. ●–●, MT vehicle; ○–○, MT 40 mg/kg; □–□, Reserpine 3 mg/kg + MT 40 mg/kg; △–△, Phentolamine 1 mg/kg.

DISCUSSION

Pain perception is not simply a function of the amount of physical injury. Rather, it is a complex state determined by multiple factors including age, sex, environment, and multiple psychological factors. Thus, pain is a subjective parameter which expresses a large variability among individuals. However, in animal tests only behavioural responses subsequent to a painful stimulus are monitored. The different animal models, such as hot-plate, writhing, and tail-flick test, were employed in our experiments to evaluate the analgesia of MT. The results showed that MT (40–60 mg/kg) produced significant analgesic action in each of the above models, which depended on dose and time of administration of MT. MT had a slower onset and a longer duration of effect in contrast to that of pethidine. The peak effect appeared 1 h after MT was administered. The ED_{50} values of MT analgesia was 34.78 mg/kg, similar to that of pethidine (34.66 mg/kg) estimated by probit analysis. These data suggest that MT can antagonize nociception caused by several factors, and the analgesic potency was equivalent to that of pethidine. The analgesia of MT was also induced by icv injection, which had a faster onset (15 min after injection) and a lower dose (0.25 mg/kg) in contrast to ip injection. These factors suggest that MT-induced analgesia is mediated through the central nervous system (CNS).

A lower dose of MT (10 mg/kg) could enhance the analgesia of pethidine. Treatment of mice with MT gave a dose- and time-dependent analgesia, which could be reversed by the opiate antagonist naloxone (10 mg/kg). beta-EP content of the hypothalamus and pituitary was decreased significantly 1 h after ip MT (100 mg/kg). Immunoenhancing and anti-stress effects of MT were abolished by naltrexone, an opioid antagonist [Maestroni

TABLE 5. Effect of MT and Morphine on Macrophages Function of Mice After Tolerance Appearing (N = 10, X ± SD)

Group	Dose (mg/kg)	K value ($\times 10^{-3}$)	α value
MT vehicle	—	84.31 ± 25.60	4.28 ± 0.40
MT	700	144.63 ± 30.23**	6.45 ± 0.78*
Morphine	60	76.92 ± 21.19*	3.94 ± 0.29*

*$P < 0.05$, **$P < 0.01$ compared with MT vehicle.

TABLE 4. Effect of $CaCl_2$, Verapamil, and EGTA on the Analgesic Action of MT (N = 10, X ± SD)†

Group	Dose (mg/kg)	Pain threshold(s) Before administration	Pain threshold(s) After administration
MT vehicle	—	14.1 ± 4.0	14.2 ± 3.8
MT (ip)	40	13.9 ± 4.0	18.4 ± 5.4*
$CaCl_2$ (sc)	230	13.7 ± 4.2	15.4 ± 5.0
$CaCl_2$ (sc)+MT (ip)	230+40	13.9 ± 4.3	14.0 ± 5.0
MT (ip)	10	14.1 ± 3.9	14.3 ± 3.8**
Ver (sc)	15	14.3 ± 4.1	15.1 ± 4.9
EGTA (sc)	180	13.6 ± 4.2	14.7 ± 3.9
MT (ip)+Ver (sc)	10+15	14.2 ± 4.0	19.7 ± 5.9***
MT (ip)+EGTA (sc)	10+180	13.7 ± 4.2	23.6 ± 7.3****

†Ver: verapamil.
*$P < 0.05$, compared with group MT vehicle.
**$P < 0.05$ compared with MT (40 mg/kg).
***$P < 0.05$, ****$P < 0.01$ compared with MT (10 mg/kg).

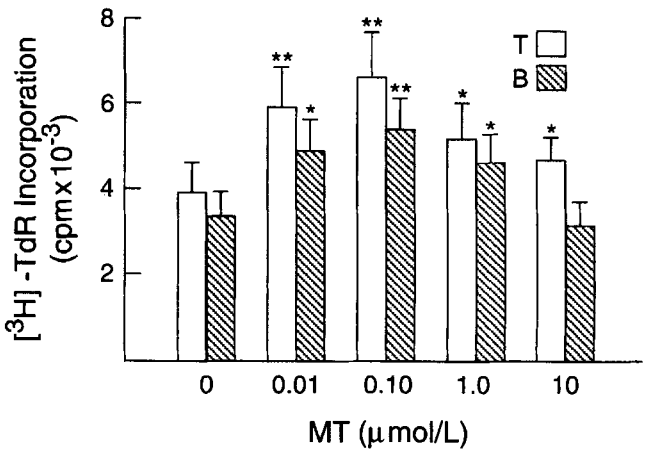

Figure 7. Effects of MT on conA- and LPS-induced proliferation of splenocytes in C57BL/6J mice, X ± SD, N = 5. *$P < 0.05$, **$P < 0.01$, compared with control, [^{3}H]-TdR:[^{3}H]-deoxythymidine.

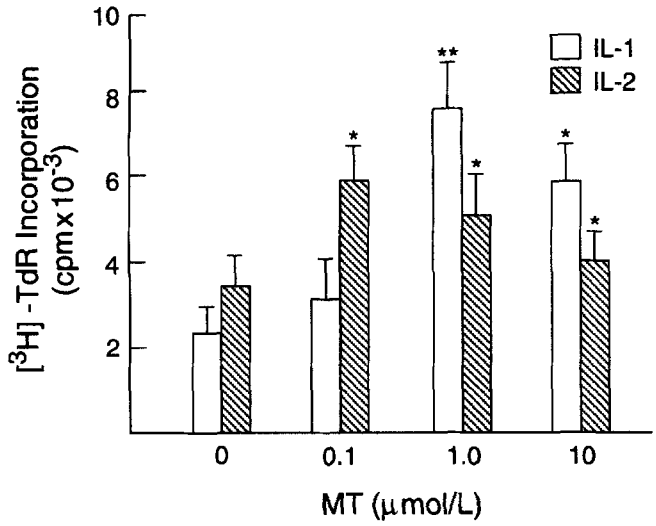

Figure 8. Effects of MT on ConA-induced IL-2 production by splenocytes and LPS-induced IL-1 production by macrophages. X ± SD, N = 5, *P < 0.05, **P < 0.01, compared with control.

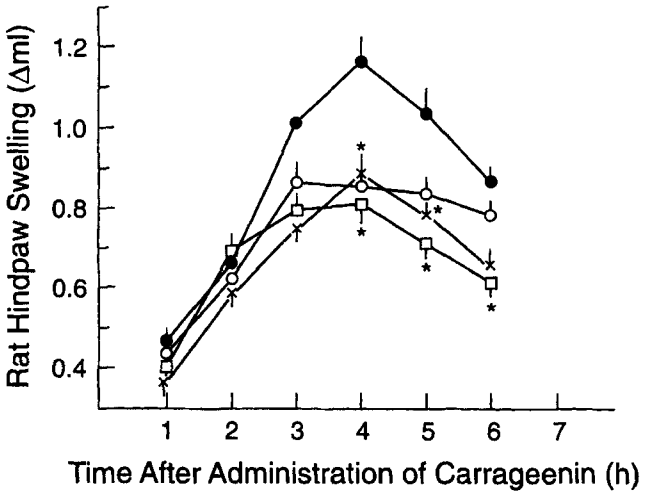

Figure 9. Edema of rat's hindpaw induced by hypodemic injection of carrageenin. MT was administered 30 min before hypodermic injection of carrageenin. (●) normal saline; (○) MT 10 μg/kg; (□) 200 μg/kg; (X) MT 4,000 μg/kg. X ± SD, N = 12, *P < 0.05, compared with normal saline.

et al., 1987]. Opioid peptides can mimic the effects of MT [Maestroni et al., 1989]. On the other hand, there is considerable evidence suggesting that opioids modulate pineal function: two of the genes encoding opioid peptides (POMC and proenkephalin) are expressed in the pineal. Furthermore, exogenously administered opioid agonists increased the circulating level of the pineal hormone MT [Esposti et al., 1988], whereas naloxone attenuated the normal nighttime elevation of MT [Lowenstein et al., 1984]. Opioid receptors located in the pineal gland play an important role in regulating pineal metabolism. These may reflect the interaction of opioids and pineal gland. In an effort to define specifically the neurotransmitters involved in nociceptive threshold, we have administered monoamine receptor antagonist and monoamine transmitter uptake inhibitors. For example, phentolamine or reserpine administered to block receptors for norepinephrine and to exhaust 5-HT and norepinephrine, respectively, attenuated the antinociceptive effects of MT. These findings showed a connection of the antinociception of MT with the descending tryptaminergic or noradrenergic pathways. The primary mechanism of MT analgesic action was analysed with drugs that modulate Ca^{2+} mobilization and use. $CaCl_2$ (230 mg/kg) antagonized the analgesia of MT, whereas the Ca^{2+} channel antagonist verapamil or the chelator EGTA enhanced the analgesia of MT. These observations suggest that the analgesia of MT may be related to Ca^{2+} entry through Ca^{2+} channels.

There was no evidence of tolerance after the administration of moderate doses of MT (40 mg/kg, qd, × 7 days) in mice hot-plate, and in large doses of MT (350 mg/kg, sc), no tail-erecting was found. In the above conditions, the immune function of mice was not affected, in contrast to narcotic drugs. It needs to be determined whether MT has tolerance and dependence in other species. MT (10 μg/kg or 0.01–10 μmol/L) could enhance ConA- and LPS-induced proliferation of splenocytes as well as the production of IL-1 and IL-2 in vitro, particularly in rats with adjuvant arthritis. It is well known that

TABLE 6. Effect of MT on Immune Function in AA Rats†

Group	ConA-induced T cell Proliferation (SI)	LPS-induced B cell Proliferation (SI)	IL-1 activity (comp × 10^{-3})
Normal	20.8 ± 4.3	17.4 ± 5.1	4.2 ± 0.7
AA rats	6.3 ± 0.7	16.5 ± 1.3	10.5 ± 1.3
MT	10.4 ± 2.4*	2.42 ± 0.8*	5.8 ± 1.1*

†MT (10 μg/kg) was injected ip to 16:00 h on day 4 after injection of CFA for 10 days; day 30 rats were killed for this experiment. X ± SD, N = 4–5. SI: Stimulation Index; CFA: complete Freund's adjuvant.
*P < 0.05 compared with AA rats.

TABLE 7. Effects of MT on Secondary Inflammatory Reaction of AA Rats†

Group	Inflammatory swelling (Δml) Day 20	Day 24
AA rats	0.64 ± 0.20	0.81 ± 0.12
MT	0.39 ± 0.11**	0.29 ± 0.05**

†Rats were treated with MT (10 μg/kg) for the control, the same amount of saline solution was injected ip once a day for 10 days at 16:00 h starting on day 4 after injection of CFA, then observed on day 20, day 24, respectively. X ± SD, N = 8–10.
**P < 0.01, compared with AA rats.

opiate receptors exist on lymphocytes, and opiate and opiate peptides regulate lymphocyte function; on the other hand, some immune cells are actually capable of secreting beta-EP. It follow that the analgesic and immunmodulatory effects of MT may be related to opioid mechanisms besides a direct action on lymphocytes.

Most human pain appears to be associated with the inflammatory process. Perhaps because of the test system used in pharmacological experiments, clear-cut distinctions are made between drugs with analgesic effects and those with antiinflammatory ones; nonsteroid antiinflammatory drugs (NSAIDS), as a group, are both analgesia and antiinflammation. In view of this, the observation on the antiinflammatory effects of MT, administered at very low doses of 10–1,000 μg/kg, indicates that MT inhibited the swelling of hindpaw induced by carrageenin and complete Freund's adjuvant in rat. These observations suggest that MT is an important antiinflammatory agent.

The analgesic drugs commonly used can be divided into two major groups: group I, the NSAIDS (such as aspirin), which produce analgesia through their ability to alter the prostaglandin system. Group II, the narcotic type analgesics (such as morphine), which bind to discrete opiate receptors and activate an endogenous pain suppression system. The properties of morphine analgesia are that it is capable of producing effective analgesia over a wide range of doses, which may, however, produce undesirable effects even after a single dose. In addition, it is easy for morphine to produce tolerance and dependence accompanied with decreased immune function after repeated use. Although the use of NSAIDS appears to produce no tolerance and dependent, it is only effect on chronic pain and many sideeffects limit its clinical application. The choice of a specific drug with high potency as well as the ability to increase immune function without tolerance and physical dependence is clinically important. Our studies indicate that MT possesses marked antiinflammatory, immunoregulatory, and analgesic effects, while no evidence indictes that MT has significant adverse effects as demonstrated in a number of published articles.

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