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STUDY OF ANTINOCICEPTIVE EFFECT OF MELATONIN ALONE AND IN COMBINATION WITH PETHIDINE IN SWISS ALBINO MICE

Kusum Kumari, Kumari Pallavi * and Keshav Kumar

Department of Pharmacology, Rajendra Institute of Medical Sciences, Ranchi - 834009, Jharkhand, India.

Keywords:

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Correspondence to Author:
Dr. Kumari Pallavi

Senior Resident,
Department of Pharmacology,
Rajendra Institute of Medical Sciences, Ranchi - 834009,
Jharkhand, India.

E-mail: dr.pallavi3105@gmail.com

ABSTRACT: Pain is the most common reason for physician consultation. Among the currently available analgesic drugs, opioids are the most efficacious but have many unacceptable adverse effects. Melatonin has been found to have antinociceptive potential in many animal and clinical studies that need further evaluation. The study aimed to assess the antinociceptive effect of melatonin alone and in combination with pethidine. Twenty Swiss albino mice were divided into four groups, each containing five animals receiving respective drugs. Tail flick and tail clip methods were used to assess antinociceptive effect of the drug. The reaction time of each animal in each group was taken at 0 min, 30 min, 60 min, 90 min and 120 min and the experiment was conducted on day 0, day 7, day 14 and day 28. Mean reaction time averaged over day 0, day 7, day 14 and day 28 was used to make inter-group comparisons. The mean reaction time increased in all groups except group I, in both tail flick and tail clip methods suggesting an antinociceptive effect of melatonin. The mean reaction time of a combination of melatonin and pethidine was much higher than that of either pethidine alone or melatonin alone, suggesting that melatonin potentiated the effect of pethidine. The antinociceptive effect of melatonin in the dose used in the present study was comparable to that of melatonin, and melatonin potentiated antinociceptive effect of pethidine.

INTRODUCTION: Pain is defined as an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage [1]. Pain is the most common reason for physician consultation in most countries. It is a major symptom of many medical conditions and can interfere with a person's quality of life and general functioning. Those who experience pain can experience acute, chronic, or intermittent pain.

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Pain is an enormous global health problem. Globally, it has been estimated that 1 in 5 adults suffer from pain and WHO has estimated that 1 in 10 adults are diagnosed with chronic pain each year. The four largest causes of pain are cancer, osteoarthritis, rheumatoid arthritis, operations and injuries, and spinal problems, making the etiology of pain a complex, trans-disciplinary affair.

Several epidemiological studies from different countries have reported widely varying prevalence rates for chronic pain, ranging from 12 to 80% of the population [2]. An Indian epidemiologic study identified point prevalence of chronic pain in India across different cities of India. The overall point prevalence of chronic pain was 13%. Respondents with moderate and severe chronic pain were 37% and 63%, respectively. Pain in knees (32%), legs

(28%), and joints (22%) was the most prevalent [3]. Historically, the pain has been treated by psychological techniques, physical methods (surgical intervention, electrical stimulant, pressure, cold, heat, counter-irritant, acupuncture), and by drugs. Acute pain is usually managed with medications such as analgesics and anaesthetics. However, chronic pain management is much more difficult. It may require the coordinated efforts of a pain management team, which typically includes medical practitioners, clinical pharmacists, clinical psychologists, physiotherapists, occupational therapists, physician assistants, and nurses. Overall, analgesic medications are the first line of treatment in pain management [4, 5].

Currently available treatments for pain are NSAIDs, opioid analgesics and some other classes of drugs. NSAIDs have side effects, including gastrointestinal ulceration and bleeding, making them unsafe for long-term clinical use [6]. Opioids are the most potent pain-relieving drugs currently available, having the broadest range of efficacy and providing the most reliable and effective method for rapid pain relief. But they are associated with several side effects like sedation, respiratory depression, pruritus, constipation and dependence, limiting the use of these drugs [7].

This led to intensive research for a compound without these side effects. The intensity of pain sensation exhibits marked day and night variations. Since the intensity of pain perception is low during dark hours of the night when melatonin levels are high, this hormone has been implicated as one of the prime antinociceptive substances. Several studies have examined the antinociceptive role of melatonin in acute, inflammatory, and neuropathic pain in animal models [8, 9].

Sleep disturbance has been seen as associated with pain [10]. Various studies on the antinociceptive effect of melatonin have contradictory results and warrant further evaluation [11]. So, this study was conducted to evaluate the antinociceptive effect of melatonin and compare the same with that of pethidine.

Aims and Objectives:

1. To evaluate the antinociceptive effect of melatonin in Swiss albino mice.
2. To compare the antinociceptive effect of melatonin with pethidine in Swiss albino mice.
3. To evaluate the antinociceptive effect of melatonin in combination with pethidine in Swiss albino mice.

MATERIALS AND METHODS: Healthy Swiss albino mice were procured from an authorized supplier, and mice weighing between 20-40 g were taken for the present study. The animals were kept in clean and dry cages with a 12 h: 12 h light-dark cycle at normal room temperature and humidity. They were acclimatized to the available housing condition in the cages for 2 weeks before using for the experiment. They were fed with a standard laboratory diet consisting of soaked black gram, maize, and water was given *ad libitum*.

Arrangements were made to ensure regular cleaning of cages and disposal of excreta and urine. The animals were inspected frequently to rule out any infection. The institutional animal ethics committee approved the experimental protocol, and the whole experiment was conducted according to the guidelines and ethical norms approved by Institutional Animal Ethics Committee (Reg No. 1104/GO/Re/S/07/ CPCSEA).

Place of Study: The study was conducted in a post-graduate research laboratory, Department of Pharmacology, RIMS, Ranchi.

Study Groups: For this study, 20 Swiss albino mice were taken and grouped randomly into 4 groups, 5 in each group. The mice were kept in four animal cages. All the cages were appropriately labelled. Animals in each cage were also labelled separately and colour coded with the help of a permanent marker. The animals were weighed with the balance. All the groups received the respective drugs by intra-peritoneal (i.p.) route through a disposable syringe.

Group	No of Animals	Drug and dose
I	5	Normal saline(10ml/kg-I.P)
II	5	Pethidine hydrochloride(6mg/Kg-I.P)
III	5	Melatonin (50mg/kg- I.P)
IV	5	Pethidinehydrochloride (6mg/Kg-I.P) + melatonin (50mg/kg- I.P)

Inclusion Criteria:

- Swiss albino mice of either sex.
- Weight between 20-40 g.
- Healthy and active in their cage.

Exclusion Criteria:

- Weight<20 g &>40 g.
- Diseased and Inactive.

Drugs:

1. Melatonin - 1gm powder.
2. Pethidine hydrochloride -2ml ampoule (100mg/2ml).

Calculation of Dose: Wherever necessary dose of the drug was calculated using human to mice conversion factor based on body surface area.

Melatonin: The dose of melatonin used for this study was 50mg/kg body weight of mice. So, the dose for a 20 g mouse will be 1mg. 50 mg of the powder (purity≥98%) was dissolved in 5 ml of normal saline to have a 10mg/ml strength.

So, 0.1 ml of the prepared drug was injected into 20 gm mice. The volume of melatonin solution was adjusted according to the body weight of the mice during i.p injection.

Pethidine Hydrochloride: The daily human dose of pethidine for pain is 50 mg/day for an average 70 kg adult. It means by multiplying 50 with 0.0026 (a human-to-mice conversion factor), the daily dose for 20 g mice will be 0.13 mg. 2 ml of injection pethidine (the ampoule containing 100 mg/2ml) was diluted in 98 ml of normal saline to have a strength of 1mg/ml. So, 0.13 ml of the prepared drug was injected into 20 gm mice. The volume of the pethidine solution was adjusted according to the body weight of the mice during i.p. injection.

Equipment and Chemicals: Tail flick analgesiometer, Artery clip, Disposable syringes, Measuring jar, Glass beaker, animal weighing balance, Cotton, Stopwatch, Normal saline, Spirit, Betadine.

Study Duration: The total duration of the experiment was 42 days, including 14 days of acclimatization of animals. Mice were given respective treatments at 1-week intervals, *i.e.*, on days 0, 7, 14, 21 & 28. Day 15 was taken as day 0.

Tests used to show Antinociceptive Effects were:

1. Tail Flick Method Using the Analgesiometer.
2. Haffner's Tail clip method.
3. Tail Flick Method Using the Analgesiometer.

Principle: Analgesia was measured using a modified method of D' Amour and Smith called as tail flick method using an analgesiometer. Applying thermal radiation to an animal's tail provokes the tail's withdrawal by a brief vigorous movement. The reaction time (time taken by the animal to withdraw its tail from the hot wire) of this movement was recorded.

Methods: At a time, one animal was held in a restrainer so that the tail lies over the nichrome wire of analgesiometer at a distance of 1.5cm. The strength of the current passing through the naked nichrome wire was kept constant at 5 amps. The site of application of the radiant heat in the tail was maintained at 2.5 cm, measured from the root of the tail. Reaction time was noted by using a stopwatch. The cut-off time of 15 sec was taken, and the tail was removed from the source of heat to avoid any tissue damage if there is no tail flicking. Normal reaction time was noted in each animal before starting the experiment. These readings were taken as reaction time at 0 minutes. Following the initial reading, all animals received the respective drugs intra-peritoneally, and the effects were measured after 30, 60, 90 and 120 minutes in the tests. The experiment was conducted on day 0, day 7, day 14 and day 28.

Haffner's Tail clip Method

Principle: By placing an artery clip at the root of the tail, a mechanical stimulus is given to the animal, and its shows the response in the form of biting the clip or tail at the position of the clip.

Methods: An artery clip was placed at the root of the tail of mice to apply a noxious mechanical stimulus. A reaction time between the application

of the clip and response was noted by the stopwatch. Normal reaction time was noted in each animal before giving any treatment. These readings were taken as reaction time at 0 minutes. Following the initial reading (called as 0 min) all animals received the respective drugs intra-peritoneally, and the effects were measured after 30, 60, 90, and 120 minutes in the tests. The experiment was conducted on day 0, day 7, day 14, and day 28. Cut off time of 15 seconds was taken & clip was removed to avoid any tissue damage.

Statistical Analysis: Reaction time was recorded in Microsoft Excel sheets for further evaluation. Statistical analysis of data was carried out using IBM SPSS version 20 software which was employed for analysis of variance (ANOVA). Post hoc analysis was done by using Tukey's HSD (honestly significant difference) test.

RESULTS: The followings are observations of the Tail flick and Tail clip methods.

TABLE 1: COMPARISON OF MEAN REACTION TIME (IN SEC) AMONG GROUP I, II, III AND IV BY TAIL FLICK METHOD AND TAIL CLIP METHOD

Group	Mean±SD									
	Tail flick method					Tail clip method				
	0 min	30 min	60 min	90 min	120 min	0 min	30 min	60 min	90 min	120 min
I	5.85± 0.26	6.00± 0.22	6.12± 0.36	6.20± 0.35	6.19± 0.32	4.06± 0.21	4.34± 0.28	4.44± 0.29	4.75± 0.32	4.54± 0.25
II	6.26± 0.22	7.4± 0.20	10.02± 0.62	8.33± 0.41	7.24± 0.40	4.60± 0.18	5.88± 0.33	7.61± 0.43	6.49± 0.32	5.65± 0.29
III	6.09± 0.05	7.09± 0.29	8.84± 0.54	7.53± 0.32	6.59± 0.24	4.27± 0.18	5.44± 0.38	6.99± 0.36	5.97± 0.39	4.94± 0.35
IV	6.24± 0.22	8.19± 0.29	11.48± 0.60	9.18± 0.61	7.98± 0.21	4.70± 0.26	7.08± 0.43	8.59± 0.40	7.16± 0.18	6.18± 0.20

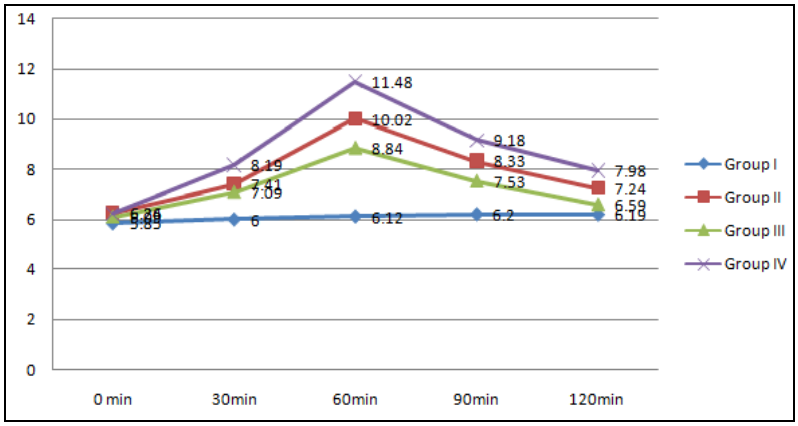

FIG. 1: LINE DIAGRAM SHOWING COMPARISON OF MEAN REACTION TIME (IN SEC) AMONG GROUP I, II, III AND IV BY TAIL FLICK METHOD

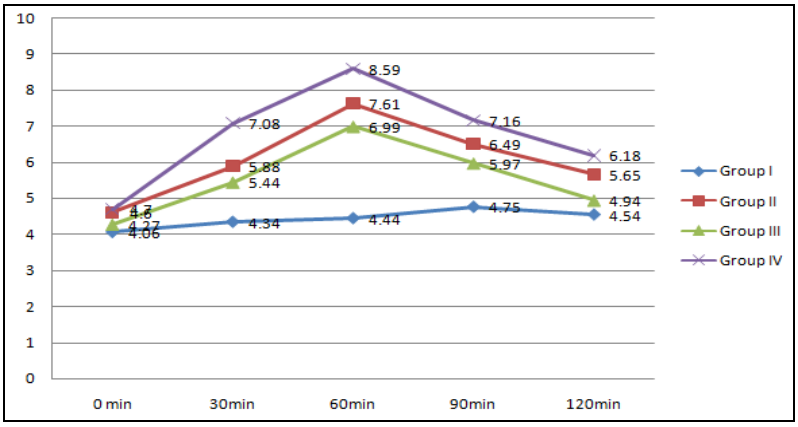

FIG. 2: LINE DIAGRAM SHOWING OVERALL COMPARISON OF MEAN REACTION TIME (IN SEC) AMONG GROUP I, II, III AND IV BY TAIL CLIP METHOD

Table 1 & **Table 2** shows there is no significant variation in mean reaction time in vehicle-treated group (Group I) both in the tail flick and tail clip methods. Further, pethidine shows maximum antinociceptive effect at 60 min, and then its effect gradually decreases. Melatonin also shows maximum antinociceptive effect at 60 min and then its effect gradually decreases. Furthermore, the combined effect of melatonin & pethidine is maximum at 60 min and then its effect gradually decreases.

TABLE 2: COMPARISON OF MEAN REACTION TIME (IN SEC) BETWEEN GROUP I AND GROUP III BY TAIL FLICK AND TAIL CLIP METHODS

Group	Tail flick method					Tail clip method				
	0 min	30 min	60 min	90 min	120 min	0 min	30 min	60 min	90 min	120 min
I	5.85	6.00	6.12	6.20	6.19	4.06	4.34	4.44	4.75	4.54
III	6.09	7.09	8.84	7.53	6.59	4.27	5.44	6.99	5.97	4.94
S D	0.17	0.77	1.92	0.94	0.28	0.14	0.78	1.81	0.86	0.28
P- value	0.75	0.00	0.00	0.00	0.45	0.87	0.00	0.00	0.00	0.45

Table 2 shows that there is a significant difference in antinociceptive effect in melatonin-treated group (Group III) compared to the vehicle-treated group (Group I) at 30 min, 60 min and 90 min. Still, there is no significant difference at 0 min and 120 min in both tail flick and tail clip methods.

TABLE 3: COMPARISON OF MEAN REACTION TIME (IN SEC) BETWEEN GROUP II AND GROUP III BY TAIL FLICK METHOD AND TAIL CLIP METHODS

Group	Tail flick method					Tail clip method				
	0 min	30 min	60 min	90 min	120 min	0 min	30 min	60 min	90 min	120 min
II	6.26	7.41	10.02	8.33	7.24	4.60	5.87	7.61	6.49	5.65
III	6.09	7.09	8.84	7.53	6.59	4.27	5.44	6.99	5.97	4.94
S D	0.12	0.23	0.83	0.56	0.46	0.24	0.31	0.43	0.37	0.50
P- value	0.75	0.07	0.08	0.26	0.14	0.45	0.59	0.93	0.87	0.87

Table 3 shows no significant difference in the antinociceptive effect between the melatonin-treated group (Group III) and pethidine treated group (Group II).

TABLE 4: COMPARISON OF MEAN REACTION TIME (IN SEC) BETWEEN GROUP II AND GROUP IV BY TAIL FLICK METHOD AND TAIL CLIP METHODS

Group	Tail flick method					Tail clip method				
	0 min	30 min	60 min	90 min	120 min	0 min	30 min	60 min	90 min	120 min
II	6.26	7.41	10.02	8.33	7.24	4.6	5.87	7.61	6.49	5.65
IV	6.24	8.19	11.48	9.18	7.98	4.7	7.08	8.59	7.16	6.18
S D	0.01	0.55	1.03	0.60	0.52	0.07	0.85	0.69	0.47	0.37
P- value	0.75	0.07	0.00	0.01	0.04	0.87	0.00	0.04	0.15	0.87

Table 4 shows a significant difference in the antinociceptive effect between pethidine treated group (Group II) and Group IV (combination of melatonin & pethidine) at 60 min, 90 min and 120 min in tail clip method and at 30min and 60min tail clip method.

TABLE 5: COMPARISON OF MEAN REACTION TIME (IN SEC) BETWEEN GROUP III AND GROUP IV BY TAIL FLICK AND TAIL CLIP METHODS

Group	Tail flick method					Tail clip method				
	0 min	30 min	60 min	90 min	120 min	0 min	30 min	60 min	90 min	120 min
III	6.09	7.09	8.84	7.53	6.59	4.27	5.44	6.99	5.97	4.94
IV	6.24	8.19	11.48	9.18	7.98	4.70	7.08	8.59	7.16	6.18
S D	0.11	0.78	1.86	1.16	0.98	0.30	1.16	1.13	0.84	0.88
P- value	1.00	0.00	0.00	0.00	0.00	0.14	0.00	0.01	0.03	0.00

Table 5 shows a significant difference in antinociceptive effect in melatonin-treated group (Group III) and Group IV (combination of melatonin & pethidine) at 30 min, 60 min, 90 min, and 120 min.

DISCUSSIONS: It was found that, after drug administration, the reaction time started to increase from baseline (at 0min), and peak effect was seen at 60 minutes, then it started to decrease at 90 min. in almost all the groups except in group I in both the methods, *i.e.,* tail flick method and tail clip method **Table 1** & **2**, **Fig. 1** & **2**. This finding suggests the test drugs *i.e.,* melatonin has an antinociceptive effect. This finding was consistent with the studies conducted by Wang *et al.* [12] who found the analgesic effect of melatonin in both experimental and clinical studies. They found that melatonin exhibits an analgesic effect in a dose-dependent manner.

When compared with pethidine (Group II), melatonin (Group III) had slightly lower mean reaction time at all points of time (*i.e.,* at 0 min, 30 min, 60 min, 90 min and 120 min) both in the tail flick method and tail clip method **Table 3** but the difference was not statistically significant, suggesting the antinociceptive effect of melatonin in the dose used in the present study was comparable to that of pethidine. The mean reaction time of a combination of pethidine and melatonin (Group IV) was much higher than that of pethidine alone (Group II) in both methods. The difference was statistically significant at 60 min, 90 min and 120 min in the Tail flick method and at 30 min and 60 min in the tail clip method. **Table 4** suggests that melatonin potentiated the antinociceptive effect of pethidine.

The mean reaction time of a combination of pethidine and melatonin (Group IV) was much higher than that of melatonin alone (Group III) in both methods. The difference was statistically significant all the time except at 0 min in both tail flick and tail clip methods **Table 5**, suggesting that melatonin potentiated antinociceptive effect of pethidine. This finding is consistent with that of studies conducted by Sheng-Hsiung L*et al* [13] which compared melatonin with opioids and found a synergistic effect between melatonin and opioids. They found melatonin potentiates the antinociceptive effect of opioids and reduces side effects of opioids, such as respiratory depression, constipation, etc., including tolerance and dependence. Several mechanisms have been proposed by which melatonin exerts its antinociceptive influence. One of the most important mechanisms of action is an activation of melatonin receptors, termed MT1 and MT2, distributed in important regions in pain control, such as lamina I-V and X of the spinal cord, thalamus, hypothalamus, spinal trigeminal tract and trigeminal nucleus *i.e.* located in both peripheral and central nervous system [13]. Activation of melatonin receptors leads to a Gi-protein-mediated decrease of cyclic AMP levels resulting in no activation of protein kinase A and hence no response. It also inhibits voltage-gated Ca^{2+} channels at the presynaptic nerve terminal leading to a decreased influx of Ca^{2+} hence decreased release of neurotransmitters and no response [14].

The activation of melatonin receptors also activates K^+-channels which inhibit an action potential firing in neurons [15]. Several other second messenger molecules like cGMP, diacylglycerol, inositol triphosphate and arachidonic acid are regulated by melatonin receptors [16]. MT receptors can transmit signals through the pertussis toxin-sensitive Gi/o protein and delivered to second messenger systems or through Gq/11-phospholipase C (PLC) and PKC-dependent mechanism to modulate Ca^{2+} signalling [17]. Melatonin modulates against Ca^{2+} influx via desensitization of transient receptor potential vanilloid type 1 and melastatin type 2 (TRPV1 and TRPM2) [18]. Accumulating evidence demonstrates that the NO/NOS system plays an important role in animal models' initiation and maintenance of nociceptive response.

The enhanced levels of NO production and NOS expression are inhibited by melatonin administration in various nociceptive states [19]. The opioid system is another supposed to be involved in the antinociceptive effect of melatonin. The facts could be evidence that naloxone, an opioid receptor antagonist, reversed the antinociceptive effect of melatonin [20].

Studies have shown that melatonin accelerates norepinephrine transmission and activates α1- and β-adrenoceptors. Moreover, activation of the noradrenergic descending pathway inhibits the activities of the spinal cord nociceptive receptors, such as α^2-adrenoceptors [21]. Melatonin binding to nuclear receptors has been demonstrated. Some of these binding sites were identified as belonging to retinoid orphan-related receptors like RZRα and

RZRβ, and they are present in the central and peripheral nervous systems [22]. Thus, the exact mechanism by which melatonin exerts its analgesic actions remains to be clarified. Studies so far conducted on antinociceptive effect of melatonin have contradictory results. Most of the studies showed antinociceptive effect of melatonin in a dose-dependent manner, *i.e.,* having an antinociceptive effect in high doses. However, some studies had the effect even in a lower dose. Many animal and clinical studies suggested that melatonin has anti- nociceptive effect for both acute pain (*viz* inflammatory, post-operative pain, *etc*.) and chronic pain (*viz* neuropathic pain, inflammatory bowel syndrome, fibromyalgia, migraine, *etc*.) while many others found it effective in only chronic pain.

A systematic qualitative review of eight randomized controlled trials with perioperative melatonin reported inconsistent and limited evidence regarding its analgesic effects [23]. Another systematic review and meta-analysis of eight randomized controlled trials with perioperative melatonin concluded that its analgesic effects were uncertain due to the profound heterogeneity [24]. A recent systematic review and meta-analysis of 19 randomized controlled trials using melatonin for various types of pain reported a significant reduction of pain [11]. A recent meta-analysis of randomized, double-blind, placebo-controlled trials conducted by Si Nae Oh *et al.* [25] found melatonin effective in chronic pain only. In the present study, the melatonin used in the given dose showed antinociceptive effects comparable with pethidine so that it could serve as an alternative or add-on to the opioid drug. Hence, many undesirable side effects, including tolerance and dependence, can be avoided. Further, melatonin exerts neuroprotection due to its antioxidant and free radical scavenging properties within the central nervous system, increasing its utility in patients with co-morbidity of pain and cognitive impairment.

Limitations of the Study:

1. Both models used in the present study were for acute pain; hence its results and conclusion cannot be projected over chronic pain models.
2. Lack of a large sample size which otherwise could have given even better results.
3. Use only a high dose of melatonin.

CONCLUSION: From observations and results of this research work, we conclude melatonin in a given dose has an antinociceptive effect. The antinociceptive effect of melatonin in a given dose is comparable with that of pethidine. Melatonin potentiates the antinociceptive effect of pethidine.

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Ethical Approval: This study was approved by the Institutional Ethics committee.

CONFLICTS OF INTEREST: There was no conflict of interest.

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