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Original article

# Involvement of serotonergic and opioidergic systems in the antinociceptive effect of ketamine-magnesium sulphate combination in formalin test in rats

Katarina Savić Vujović[a,*], Sonja Vučković[a], Dolika Vasović[b], Branislava Medić[a], Radan Stojanović[a], Nevena Divac[a], Dragana Srebro[a], Milica Prostran[a]

[a] Department of Pharmacology, Clinical Pharmacology and Toxicology, Faculty of Medicine, University of Belgrade, Belgrade, Serbia
[b] Faculty of Medicine, University of Belgrade, Belgrade, Serbia

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ABSTRACT

*Introduction:* Ketamine and magnesium sulphate showed synergic interaction in the tail-immersion test and additive interaction in the rat formalin test. Aim of study was to evaluate the influence of serotonergic and opioidergic system of this combination in the formalin test in rats.
*Methods:* Antinociceptive activity was assessed by the formalin test in male Wistar rats (200–250 g). Antagonists (naloxone and methysergide) were administrated 5 min before and magnesium sulphate 5 min after ketamine injection. Formalin (2.5%, 100 μL) was injected into the right hind paw surface (intraplantar) of rats 5 min after ketamine/magnesium combination. Data were recorded as the total time spent in pain related behavior after the injection of formalin or vehicle (0.9% NaCl).
*Results:* In the intermediate phase of the formalin test, methysergide at a dose of 0.2 mg/kg did not have any effect, but at doses of 0.5 and 1 mg/kg it had a pronociceptive effect. Methysergide (0.2, 0.5 and 1 mg/kg) inhibited the antinociceptive effect of ketamine-magnesium sulphate combination. In the intermediate phase, naloxone at a dose of 0.2 mg/kg did not have any effect, but at a dose of 3 mg/kg it produced a pronociceptive effect. Naloxone (0.2 and 3 mg/kg) antagonized the antinociceptive effect of the ketamine (5 mg/kg)-magnesium sulphate (5 mg/kg) combination.
*Conclusion:* The results of the present study suggest that serotonergic and opioidergic systems are involved, at least in part, in the antinociceptive effect of the ketamine-magnesium sulphate combination in the model of inflammatory pain in rats.


## Introduction

Ionotropic receptors play a pivotal role in the transmission of nociceptive signals from the periphery to the central nervous system. Nociceptive impulses are transmitted in the spinal cord by unmyelinated C fibers and myelinated Aδ fibers, which lead to glutamate release in the spinal cord and to the sensation of pain [1–3]. N-methyl-D-aspartate (NMDA) receptors are located in the dorsal root ganglia of the spinal cord as well as in certain pre- and postsynaptic parts of the brain, and assume a major role in the modulation of nociceptive stimuli from the periphery [4–6]. Peripheral NMDA receptors are also included in the pathogenesis of pain. It has been shown that serotonergic pathways projecting from the dorsal raphe nucleus to locus coeruleus (LC) play a role in pain modulation and that NMDA receptors can tonically modulate serotonergic receptors on the LC [7–9].

Ketamine can facilitate endogenous pain pathways in some pain conditions. Ketamine is a NMDA receptor antagonist which is used as an adjuvant and an analgesic drug at subanestetic doses. This drug is rarely used as the only agent because of the narrow therapeutic window between analgesia and side effects such as sedation, hallucinations and psychotomimetic effects [10]. Also, it can interact with opioid receptors [11]. Ketamine can enhance opioid analgesia and can be used with other agents. Ketamine can increase the release of norepinephrine, dopamine and serotonin (monoamine neurotransmitters) and inhibit their uptake, thus potentiating the inhibitory descending nociceptive pathways [12–14].

Magnesium is a physiological non-competitive NMDA receptor antagonist which blocks the channel in a dose-dependent manner. It has been reported that magnesium can increase analgesia

* Corresponding author.
*E-mail address:* katarinasavicvujovic@gmail.com (K. Savić Vujović).

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induced by opioids, general and local anesthetics in different models of pain. The main mechanism of action through which magnesium produces central effects is blockage of the calcium channels associated with NMDA receptors [15–17].

Ketamine and magnesium can interact in additive, antagonistic and supraaditive manners to produce analgesia or anesthesia [18–20]. Previously we demonstrated that the ketamine-magnesium sulphate combination shows synergic interaction in the tail-immersion test and additive interaction in the rat formalin test [10]. Therefore, the aim of present study was to evaluate the influence of serotonergic and opioidergic systems on the antinociceptive effect of the combination of ketamine-magnesium sulphate in the formalin test in rats.

## Material and methods

### *Animals*

Experiments were performed in adult, 2 months old, male Wistar rats (n = 150) weighing between 200 and 250 g obtained from the Military Medical Academy (Belgrade, Serbia). Before the beginning and during the experiments, animals were housed in polycarbonate cages (42.5 × 27 × 19 cm) in groups of three per cage. The living conditions were maintained constant with a temperature of 22 ± 1 °C °C, humidity of 60% and a 12 h light/dark cycle (with lights on at 8:00 a.m.). The animals had ad libitum access to food (pellets of standard rodent diet, obtained from the Veterinary Institute Subotica, Serbia) and water (tap water). In order to avoid diurnal variation, all experiments were conducted at the same time of the day (between 8:00 a.m. and 2:00 p.m.) by the same experimenter, continuously. During testing animals were unrestrained. Each animal was used only once and sacrificed at the end of the experiments by intraperitoneal (*ip*) injection of sodium thiopental (200 mg/kg).

### *Ethical statement*

The methodology used in our investigation was approved by the Ethics Committee for Animal Research and Welfare of the Faculty of Medicine, University of Belgrade (Permit N°3416/2). Ethical Council for the Protection of Experimental Animals of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia approved all experiments. Additionally, all procedures were in accordance with the Animal Welfare Law of the Republic of Serbia and the International Association for the Study of Pain (IASP) Guidelines for the Use of Animals in Research.

### *Drugs and their administration*

Ketamine (Inresa Arzneimittel GmbH, Freiburg, Germany) and magnesium sulphate (Zorka, Šabac, Serbia) were dissolved in 0.9% NaCl and injected subcutaneously (*sc*) and intraperitoneally (*ip*), respectively, in total volume of 2 ml/kg. Magnesium sulphate was administered 5 min after ketamine injection. Formalin (2.5%, 100 μL) was injected into the right hind paw surface (intraplantar *ipl*) of rats 5 min after ketamine/magnesium sulphate combination. Antagonists (naloxone and methysergide) (Sigma-Aldrich Chemical Co., St Louis, MO., SAD), were administrated *sc* 5 min before ketamine. In order to analyze any possible effect on the antinociception, the same volume of 0.9% NaCl was administered to the control group.

### *Formalin test*

100 μL of 2.5% formalin was injected into the plantar surface of the right hind paw of rats using a microliter syringe and a 29 gauge needle. Data were reported (in 9 blocks of 5 min) by measuring the total time spent in pain related behavior (the injected paw was elevated and without any contact with surface; animal licked or bit the injected paw) after the injection of formalin or vehicle. It was measured the nociceptive time during the acute phase (0–5 min), intermediate (5–10 min) and second phase (10–45 min) after formalin injection.

### *Data analysis*

Results are presented in the form of bar diagrams. Each bar represents the average time (mean ± SEM) spent in nociceptive behavior for 6 animals (i.e. one group for each substance or combination of substances). Abscissa (X-axis) displays observed time interval (min) while ordinate (Y-axis) displays nociceptive reply as time animal spent in nociceptive position.

### *Statistical analysis*

Statistical analysis was performed using one way analysis of variance (ANOVA), followed by Tukey's HSD *post hoc* test. A *p* value lower than 0.05 was considered as statistically significant.

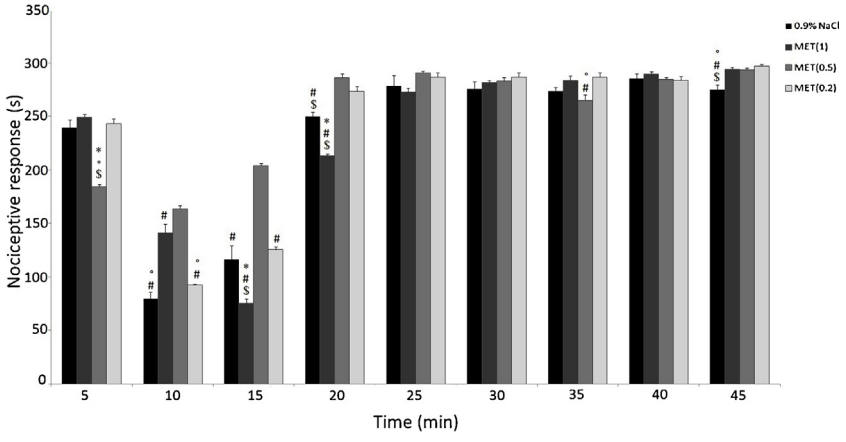


**Fig. 1.** The antinociceptive effect of methysergide in the formalin test in rats. Each point represents the mean ± SEM of the antinociceptive latency time in seconds (s) obtained in 6–8 rats. At each time interval the differences between the corresponding means were verified using one-way analysis of variance (ANOVA; F-value, *p*-value), followed by Tukey's HSD *post hoc* test where statistical significance was determined by comparing with the control (0.9% NaCl) (*$p < 0.05$ in comparison with control, °$p < 0.05$ in comparison witg MET (1), #$p < 0.05$ in comparison with MET (0.5), \$ $p < 0.05$ in comparison with MET (0.2)).

## Results

*The influence of methysergide in the formalin test in rats*

During the acute phase of the formalin test, methysergide at doses of 0.2 and 1 mg/kg did not have any influence on the nociception as compared to the control (0.9% NaCl) ($p > 0.05$). In the intermediate phase, methysergide at a dose of 0.2 mg/kg did not have any effect, but at doses of 0.5 and 1 mg/kg it produced a pronociceptive effect compared to the control (0.9% NaCl). Methysergide (0.2, 0.5 and 1 mg/kg) showed no influence on nociception in the formalin test in the period 20–40 min as compared to the control (0.9% NaCl) ($p > 0.05$) (Fig. 1). Methysergide did not produce a dose-dependent effect in the formalin test. For the following time intervals: 0–5, 5–10, 10–15, 15–20, 20–25, 25–30, 30–35, 35–40 and 45–50 min, the F- and *p*-values (ANOVA) were: [$F_{3,20} = 44.143$; $p = 0.000$], [$F_{3,20} = 55.708$; $p = 0.000$], [$F_{3,20} = 62.419$; $p = 0.000$], [$F_{3,20} = 101.938$; $p = 0.000$], [$F_{3,20} = 2.365$; $p = 0.101$], [$F_{3,20} = 1.388$; $p = 0.275$], [$F_{3,20} = 6.600$; $p = 0.003$], [$F_{3,20} = 0.827$; $p = 0.495$] and [$F_{3,20} = 16.671$; $p = 0.000$], respectively.

*The influence of methysergide on the ketamine-magnesium sulphate combination in the formalin test in rats*

When administrated *sc* 5 min before the ketamine (5 mg/kg)-magnesium sulphate (5 mg/kg) combination, methysergide (0.2, 0.5 and 1 mg/kg) inhibited the antinociceptive effect of this combination. There was statistical significance between the effects produced by different doses of methysergide when used in combination with the ketamine-magnesium sulphate combination ($p > 0.05$) (Fig. 2). For the following time intervals: 0–5, 5–10, 10–15, 15–20, 20–25, 25–30, 30–35, 35–40 and 45–50 min, the F- and *p*-values (ANOVA) were: [$F_{4,25} = 38.040$; $p = 0.000$], [$F_{4,25} = 2536.344$; $p = 0.000$], [$F_{4,25} = 252.083$; $p = 0.000$], [$F_{4,25} = 2135.566$; $p = 0.000$], [$F_{4,25} = 500.936$; $p = 0.000$], [$F_{4,25} = 495.352$; $p = 0.000$], [$F_{4,25} = 535.610$; $p = 0.000$], [$F_{4,25} = 491.597$; $p = 0.000$] and [$F_{4,25} = 282.938$; $p = 0.000$], respectively.

*The influence of naloxone in the formalin test in rats*

When administered alone, naloxone at a dose of 0.2 mg/kg did not affect nociception in the acute and intermediate phases of the formalin test (in comparison with the control). In the second phase of the formalin test, naloxone at a dose of 3 mg/kg had no influence on the nociception in the period of 20–30 min, and at a dose of 0.2 mg/kg in the period 20–25 min ($p > 0.05$) (Fig. 3). For the following time intervals: 0–5, 5–10, 10–15, 15–20, 20–25, 25–30, 30–35, 35–40 and 45–50 min, the F- and *p*-values (ANOVA) were: [$F_{2,13} = 31.753$; $p = 0.000$], [$F_{2,15} = 2132.730$; $p = 0.000$], [$F_{2,15} = 160.289$; $p = 0.000$], [$F_{2,15} = 89.484$; $p = 0.000$], [$F_{2,15} = 2.803$; $p = 0.092$], [$F_{2,15} = 5.379$; $p = 0.017$], [$F_{2,15} = 26.552$; $p = 0.000$], [$F_{2,15} = 8.119$; $p = 0.004$] and [$F_{2,15} = 25.458$; $p = 0.000$], respectively.

*The influence of naloxone on the ketamine-magnesium sulphate combination in the formalin test in rats*

Naloxone (0.2 and 3 mg/kg) antagonized the antinociceptive effect of the ketamine (5 mg/kg)-magnesium sulphate (5 mg/kg) combination when administrated *sc* 5 min before the combined drugs (Fig. 4). The effect of naloxone lasted almost all of the 5 min periods, from 0 to 50 min after ketamine-magnesium application ($p < 0.05$). For the following time intervals: 0–5, 5–10, 10–15, 15–20, 20–25, 25–30, 30–35, 35–40 and 45–50 min, the F- and *p*-values (ANOVA) were: [$F_{3,20} = 42.257$; $p = 0.000$], [$F_{3,20} = 922.613$; $p = 0.000$], [$F_{3,20} = 360.924$; $p = 0.000$], [$F_{3,20} = 2703.008$; $p = 0.000$], [$F_{3,20} = 546.739$; $p = 0.000$], [$F_{3,20} = 487.158$; $p = 0.000$], [$F_{3,20} = 616.219$; $p = 0.000$], [$F_{3,20} = 569.814$; $p = 0.000$] and [$F_{3,20} = 296.151$; $p = 0.000$], respectively.

## Discussion

Previously it was shown that in the tail-immersion test in rats, ketamine at a dose of 5 mg/kg, and magnesium sulphate at a dose of 5 mg/kg, did not produce an antinociceptive effect. As the only used agents and when applied separately they did not have an antinociceptive effect, but in combination they did [21,22]. Also, it was shown that serotonergic, GABA-ergic and noradrenergic systems are involved in the mechanism of the analgesic effect of the ketamine-magnesium sulphate combination in the acute pain test [23].

In the formalin test in rats, ketamine (5 mg/kg) had an antinociceptive effect, but magnesium (5 mg/kg) did not [10]. When ketamine was given before magnesium sulphate, the two agents interacted in an additive manner. In contrast, when magnesium sulphate was given before ketamine, the drugs interacted in an antagonistic manner. The present study examined the influence of serotonergic and opioidergic systems in the

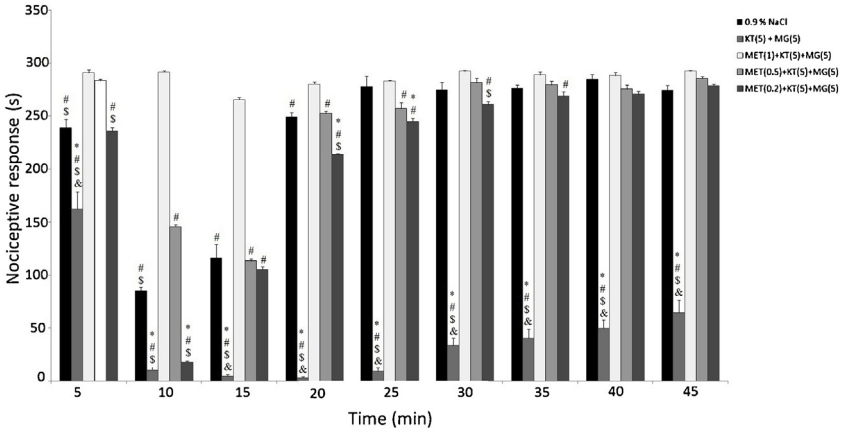


**Fig. 2.** The antinociceptive effect of methysergide-ketamine-magnesium combination in the formalin test in rats. Each point represents the mean ± SEM of the antinociceptive latency time in seconds (s) obtained in 6–8 rats. At each time interval the differences between the corresponding means were verified using one-way analysis of variance (ANOVA; F-value, *p*-value), followed by Tukey's HSD *post hoc* test where statistical significance was determined by comparing with the control (*$p < 0.05$ in comparison with control, #$p < 0.05$ in comparison with MET (1) + KT (5) + MG (5), $ $p < 0.05$ in comparison with MET (0.5) + KT (5) + MG (5), & $p < 0.05$ in comparison with MET (0.2) + KT(5) + MG (5)).

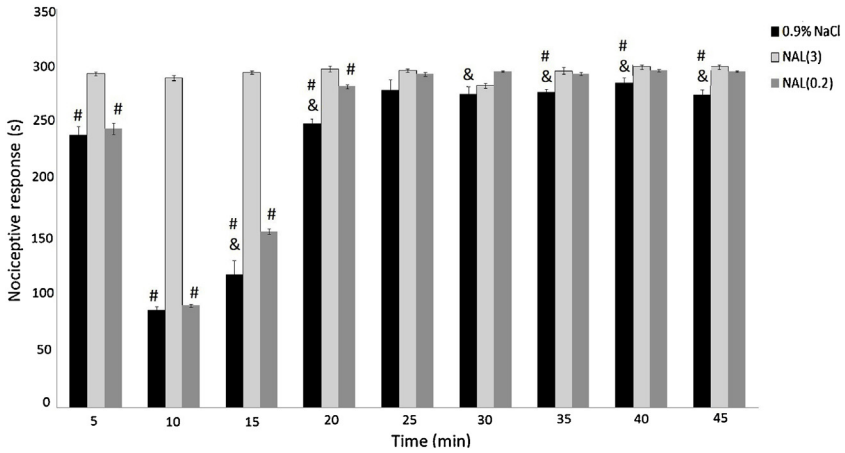


**Fig. 3.** The antinociceptive effect of naloxone in the formalin test in rats. Each point represents the mean ± SEM of the antinociceptive latency time in seconds (s) obtained in 6–8 rats. At each time interval the differences between the corresponding means were verified using one-way analysis of variance (ANOVA; F-value, *p*-value), followed by Tukey's HSD *post hoc* test where statistical significance was determined by comparing with the control (#$p < 0.05$ in comparison with NAL (3) and & $p < 0.05$ in comparison with NAL (0.2)).

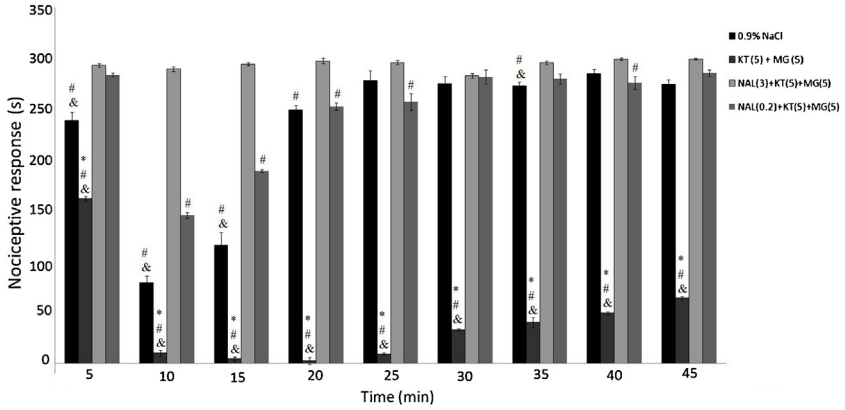


**Fig. 4.** The antinociceptive effect of naloxone-ketamine-magnesium combination in the formalin test in rats. Each point represents the mean ± SEM of the antinociceptive latency time in seconds (s) obtained in 6–8 rats. At each time interval the differences between the corresponding means were verified using one-way analysis of variance (ANOVA; F-value, *p*-value), followed by Tukey's HSD *post hoc* test where statistical significance was determined by comparing with the control (*$p < 0.05$ in comparison with control, # $p < 0.05$ in comparison with NAL (3) + KT (5) + MG (5) and & $p < 0.05$ in comparison with NAL (0.2) + KT (5) + MG (5)).

additive, antinociceptive effect of the ketamine-magnesium sulphate combination in the formalin test in rats.

Serotonin plays a role in the inhibition or facilitation of the process of transmission and perception of pain impulses. Different 5-HT receptors are present in the central and peripheral nervous systems [24]. It was shown that 5-$HT_1$ receptors are involved in the process of antinociception, whereas 5-$HT_2$ receptors have pronociceptive effects [1,25].

In the intermediate phase of the formalin test, methysergide at a dose of 0.2 mg/kg did not have any effect, but at doses of 0.5 and 1 mg/kg it had a pronociceptive effect. The intermediate phase remains unexplained. This period between phases with a nociceptive response is considered as the phase of inactivity [26]. However, several studies have shown that active inhibitory mechanisms are involved during this period [27]. Callegari et al. [28], found that the phentolamine-induced increment in the pain response during the intermediate phase was related to central pain inhibition and proposed that it was the result of interference with the noradrenergic system. Our results suggest that inhibitory serotonergic and opioidergic descending pain pathways are activated in the intermediate phase of the formalin test in rats.

In the present study, methysergide at doses of 0.2, 0.5 and 1 mg/kg, antagonized the antinociceptive effect of the combination of ketamine-magnesium sulphate in the formalin test in rats. The effects of all doses were significant from 0 to 45 min after administration of the drug combination. The finding that methysergide as a non-selective antagonist of 5-HT receptors antagonized the antinociceptive effects of the ketamine-magnesium sulphate combination suggests that the serotonergic system was involved either directly or indirectly in the antinociceptive effect of the combination. This is in correlation with previous results showing that this system is involved in the analgesic effect of this combination in the model of acute pain in rats [23].

It was concluded that ketamine can augment the endogenous anti-nociceptive system and that it exerts an antidepressant effects through activation of the serotonergic system [13,29,30]. The descending serotonergic inhibitory pathway is probably implicated in the mechanism of action of the ketamine-magnesium sulphate combination in the formalin test in rats. In addition, magnesium is well known for its NMDA receptor ion channel blocking effect, modulatory action on different ion channels and neurotransmitters release [31–35].

Ketamine acts through different mechanisms of action. It is an antagonist of the NMDA receptors but it has direct effects on δ and μ receptors [36,37]. Ketamine can modify responsiveness to opioid receptors and its analgesia was not reduced by naloxone [38]. In

our experiments, naloxone antagonized the analgesic effect of the ketamine-magnesium sulphate combination in the formalin test in rats. Our results are in agreement with the investigation that ketamine antinociception is mediated by endogenous opioids and μ- and δ-opioid receptors [11].

Interactions between ketamine and the opioid system are probably more relevant in chronic pain. Ketamine reduces opioid tolerance. Gupta et al. [8] showed that *in vitro*, ketamine prevented and reversed opioid μ-receptor desensitization through ERK1/2 phosphorylation. Magnesium at a dose of 5 mg/kg did not produce an antinociceptive effect, but in combination with ketamine, magnesium interacted in an additive manner, augmenting ketamine antinociception.

It was shown that intrathecal ketamine did not produce an antinociceptive effect in the rat [39,40]. However, some researchers concluded that intrathecal administration of ketamine can produce an antinociceptive effect through spinal opioidergic, noradrenergic and serotonergic systems [41]. The reason for the discrepancy of these results may be because of different doses, species, sex and age of animals. Kawamata et al. [39], showed that ketamine induced motor impairment in rats. Intrathecal pretreatment with yohimbine and methysergide inhibited the antinociceptive effect of ketamine, but not the ketamine-induced motor impairment.

Interphase is considered an inactive period, though recent research showed that active inhibitory mechanisms are involved in the modulation of pain during this period [42]. In the intermediate phase, naloxone at a dose of 0.2 mg/kg did not have any effect, but at a dose of 3 mg/kg it produced a pronociceptive effect. At the dose of 0.2 mg/kg, naloxone antagonized the effect of ketamine-magnesium sulphate. Again, our results imply that the inhibitory descending pathway of pain was probably activated during the intermediate phase of the formalin test in rats.

## Conclusion

The results of the present study suggest that serotonergic and opioidergic systems are involved, at least in part, in the antinociceptive effect of the ketamine-magnesium sulphate combination in the model of inflammatory pain in rats.

## Contribution

KSV conceived and designed the experiments, performed the experiments, wrote the paper. KSV and SV analyzed the data. All authors revised the manuscript and approved the final manuscript for submission.

## Conflict of interest

The authors declare that they have no conflict of interests.

## Acknowledgment

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