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Additive and antagonistic antinociceptive interactions between magnesium sulfate and ketamine in the rat formalin test

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Because ketamine and magnesium block NMDA receptor activation by distinct mechanisms of action, we hypothesized that in a model of inflammatory pain in rats the combination of ketamine and magnesium might be more effective than ketamine alone.

Antinociceptive activity was assessed by the formalin test in male Wistar rats (200–250 g). Animals were injected with 100 μL of 2.5% formalin to the plantar surface of the right hind paw. Data were recorded as the total time spent in pain-related behavior after the injection of formalin or vehicle (0.9% NaCl).

Ketamine and magnesium sulfate given separately reduced nocifensive behavior in the second phase of the formalin test in rats. When ketamine was applied after magnesium sulfate, the log dose-response curves for the effects of ketamine and the magnesium sulfate-ketamine combination revealed antagonistic interaction, and about 1.6 (CL 1.2–2.4) fold increment in ketamine dosage. A low dose of magnesium sulfate (5 mg/kg, subcutaneously) administered after ketamine increased the antinociceptive effect of ketamine by a factor of only 1.2 (CL 0.95–1.38), indicating an additive interaction. There was a 1.8-fold reduction in dosage of ketamine when ketamine was administered before rather than after the magnesium sulfate.

The present study revealed that both ketamine and magnesium reduced pain-related behavior in the second phase of the formalin test in rats. Ketamine, when administered before or after the magnesium, provided additive or antagonistic antinociceptive interactions, respectively. Whether there will be an additive or antagonistic antinociceptive interaction between ketamine and magnesium depends on the order of drug administration.

Key words: ketamine, magnesium sulfate, formalin test, interaction, rats

INTRODUCTION

The formalin model test is widely used for evaluating the effects of antinociceptive drugs in laboratory animals. Injection of formalin into the hind paw induces a biphasic pain response; the first phase is result from direct activation of primary afferent sensory neurons, whereas the second phase has been proposed to reflect the combined effects of afferent input and central sensitization in the dorsal horn (Mcnamara et al. 2007, Tjølsen et al. 1992, Pitcher and Henry 2002). Central sensitization is involved in the establishment of chronic neuropathic or inflammatory pain. The N-methyl-D-aspartate (NMDA) receptor plays a key role in mechanisms relating to central sensitization in the spinal cord (Latremoliere and Woolf 2009). Magnesium is the fourth most abundant essential ion in the human body and has a fundamental role in many cellular functions, such as storage, metabolism and energy utilization, and there is therefore increasing interest in its role in clinical medicine (Herroeder et al. 2011). Magnesium ions serve as cofactors in about 300 known enzymatic reactions in the body and in several important processes such as hormone receptor binding, gating of calcium channels, transmembrane ion flux, regulation of the adenylyl cyclase system, neuronal activity, vasomotor tone, cardiac excitability and neurotransmitter release (Schulz-Stübner et al. 2001). As magnesium blocks the NMDA receptor and its associated ion channels, it can prevent the central sensitization caused by peripheral

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nociceptive stimulation (Schulz-Stübner et al. 2001, Liu et al. 2001, Cavalcante et al. 2013). However, there are controversial results in studies in which the effects of magnesium in models of somatic inflammatory pain were investigated (Begon et al. 2002, Takano et al. 2000).

Ketamine, a dissociative anesthetic, is the most potent NMDA-receptor-channel blocker available for clinical use. Ketamine binds to the phencyclidine site when the channels are in the open activated state (Øye 1998, Quibell et al. 2011). It can also bind to a second membrane-associated site, that, decreases the frequency of channel opening (Orser et al. 1997b). Blocking NMDA receptors with ketamine reduces central sensitization and wind-up, resulting in pain reduction. Several lines of evidence indicate that at subanesthetic doses ketamine is effective against neuropathic and inflammatory pain. However, its use as the only antinociceptive drug is limited because of the high incidence of adverse effects (Shimoyama et al. 1999, Hirota and Lambert 2011, Niesters et al. 2014).

It has been reported that magnesium can either increase or decrease the antinociceptive, anesthetic or other actions of ketamine (Irifune et al. 1992, DeRossi et al. 2012, Jahangiri et al. 2013, Macdonald et al. 1991, Stessel et al. 2013). However, a statistically significant interaction (synergistic) between ketamine and magnesium was described in only three studies (Liu et al. 2001, Vučković et al. 2014, Savic Vujovic et al. 2015). In addition, Orser and others (1997a) demonstrated that low blood levels of magnesium or a magnesium-deficient diet increased the sensitivity to ketamine.

Because ketamine and magnesium block NMDA receptor activation by distinct mechanisms of action, we hypothesized that in inflammatory pain, a combination of ketamine and magnesium might be more effective than ketamine alone. Therefore, the objective of the present study was to determine the type of interaction between systemic magnesium sulfate and ketamine in the second phase of the rat formalin test, and to determine the importance of the order of drug administration.

METHODS

Subjects

The study was performed using 96 male Wistar rats (Military Farm, Belgrade, Serbia) weighing 200–250 g. The experimental animals were handled as prescribed by the Ethics Committee for Animal Research and Welfare of the Faculty of Medicine, University of Belgrade (Permit N° 3416/2). All experiments were approved by the Ethical Council for the Protection of Experimental Animals of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia, which operates 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. The animals were housed in groups of three in Plexiglas cages (42.5×27×19 cm) under standard conditions of temperature (22±1°C), relative humidity (60%) and a 12 h light/dark cycle, with lights on at 8:00 a.m. Food and water were freely available, except during the experimental procedures. The animals were fed standard rat pellets obtained from the Veterinary Institute Subotica, Serbia. The experiments were conducted by the same experimenter on consecutive days, always at the same time of the day, between 8:00 a.m. and 2:00 p.m., to avoid diurnal variation in the behavioral tests. The animals were unrestrained during testing. Each animal was used only once and was killed at the end of the experiments by an intraperitoneal (ip) injection of sodium thiopental (200 mg/kg).

Administration of drugs

Ketamine at doses of 2, 2.5 and 5 mg/kg (InresaArzneimittel GmbH, Freiburg, Germany) and magnesium sulfate at doses of 5, 15 and 30 mg/kg (Zorka, Šabac, Serbia) were dissolved in 0.9% NaCl and injected subcutaneously (sc) and intraperitoneally (ip), respectively, in a final volume of 2 ml/kg. Magnesium sulfate was administered either 5 min before or 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 sulfate. To test whether the 0.9% NaCl injection had any effect on the antinociception, the same volume of 0.9% NaCl was administered to a control group of rats.

Formalin test

Animals were injected with 100 µL of 2.5% formalin into the plantar surface of the right hind paw using a microliter syringe and a 29-gauge needle. After formalin injection, the animals were individually placed in transparent observation chambers. Data were recorded as the total time spent in pain-related behavior (the injected paw was elevated and not in contact with any surface; animal licked or bit the injected paw) after the injection of formalin or vehicle (Makau et al. 2014). The nociceptive time was calculated duringthe first phase (0–10 min) and second phase (10–45 min) after formalin injection. The recordings were performed in 9 blocks of 5 min (Makau et al. 2014).

Data analysis

The results are presented as the means ±SEM for 6–8 animals per group. The time-course of the antinociceptive responses of individual drugs and their combinations were constructed by plotting the mean time that the animal spent in pain-related behavior as a function of time. The areas delineated by the pain-related behavior and the time curves (AUC) were calculated using the trapezoidal rule. AUC was calculated for the second phase of the assay and the percentage of antinociception was calculated according to the following equation (Jiménez-Andrade et al. 2003):

Percentofantinociception(AA%)=[(AUCvehicle-AUCpost compound)/AUCvehicle]×100.

Analysis of the interaction between drugs with a high and a low antinociceptive efficacy

The interaction between ketamine (the drug that possessed a high antinociceptive efficacy and exhibited a dose-dependent effect in the formalin test) with magnesium sulfate (the drug with a low antinociceptive efficacy in the formalin test) was evaluated by the administration of a fixed, subeffective dose of magnesium sulfate with increasing doses of ketamine (Gaitan and Herrero 2002). The antinociceptive effects of ketamine were examined and the ED_{50} value obtained from the corresponding log dose-response curve. The effects of magnesium sulfate were examined for a specific dose range (based on the literature data). The lowest subeffective dose tested was chosen for the combination in experiments. In the next step, the effects of ketamine-magnesium sulfate combinations were examined. We combined fixed-dose fractions of the ED_{50} of ketamine (1 ED_{50}, 5/4 ED_{50}, 2 ED_{50}) with a fixed dose of magnesium sulfate (5 mg/kg). To determine the type of interaction, the regression log dose-response curves for ketamine alone were compared to the log dose-response curves for ketamine in the presence of magnesium sulfate. The same procedure was performed for the determination of the type of interaction between ketamine and magnesium sulfate when magnesium sulfate was administered either before or after ketamine. If there is a significant leftward shift of the log dose-response curves, the interaction between the components is supra-additive (synergistic). If the curves overlap, there is an additive interaction (Tallarida 2000). If there is a significant rightward shift

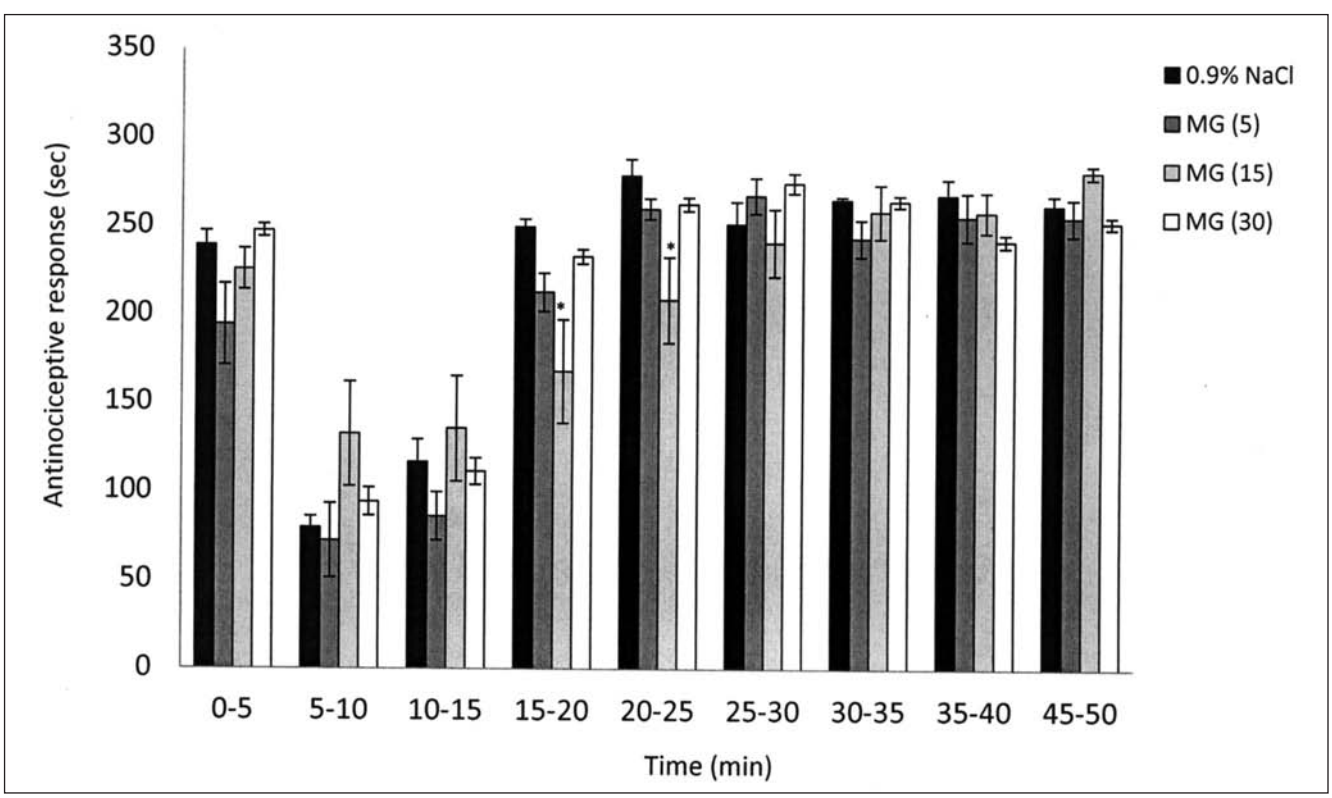

Fig. 1.The antinociceptive effect of magnesium sulfate in the formalin test in rats. Each point represents the mean ±SEM of the antinociceptive latency time in seconds (s) obtained in 6–8 rats. At each time interval the differences between the corresponding means were verified using one-way analysis 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).

of the log dose-response curves, the interaction between compounds is antagonistic.

Statistical analysis

All computations were performed according to Tallarida (2000) and Tallarida and Murray (1986). At each time interval the differences between the corresponding means were verified using one-way analysis of variance (ANOVA), followed by Tukey's HSD *post hoc* test. Two regression lines were compared by the test for parallelism and the relative potency test (Tallarida and Murray 1986). The potency ratio was considered statistically significant when 95% of the confidence limits (CL) did not overlap 1.0 (P<0.05). A *P*<0.05 was considered to be statistically significant.

RESULTS

The influence of magnesium sulfate on the formalin test in rats

Administered alone, magnesium sulfate (5 mg/kg and 30 mg/kg) did not produce any effect in comparison with control (0.9% NaCl) in the formalin test in rats (*P*>0.05) (Fig. 1). At dose of 15 mg/kg, magnesium sulfate had antinociceptive effects in the period during 15–25 min after the formalin injection (*P*<0.05). The effect of magnesium sulfate was not dose-dependent. For 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: [$F_{3,20}$=2.964; p=0.057], [$F_{3,20}$=2.024; p=0.143], [$F_{3,20}$=1.286; p=0.306], [$F_{3,20}$=4.926; p=0.010], [$F_{3,20}$=5.128; p=0.009], [$F_{3,20}$=1.437; p=0.262], [$F_{3,20}$=1.135; p=0.359], [$F_{3,20}$=1.129; p=0.361] and [$F_{3,20}$=4.062; p=0.021], respectively.

The effect of ketamine in the formalin test in rats

When administered alone, ketamine (2, 2.5 and 5 mg/kg) decreased the total time spent in pain-related behavior after the injection of formalin (*P*<0.05) (Fig. 2). Ketamine inhibited the phase 2 responses in a dose-dependent manner, but had a lower effect in phase 1. Statistical significance was observed between ketamine (2 and 2.5 mg/kg) and ketamine (5 mg/kg) in almost all 5-min periods from 20 to 45 min. For 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: [$F_{4,25}$=6.839; p=0.001], [$F_{4,25}$=1.581; p=0.210], [$F_{4,25}$=20.146; p=0.000], [$F_{4,25}$=26.856; p=0.000], [$F_{4,25}$=40.777; p=0.000], [$F_{4,25}$=41.870; p=0.000], [$F_{4,25}$=39.074; p=0.000], [$F_{4,25}$=77.629; p=0.000] and [$F_{4,25}$=124.574; p=0.000], respectively.

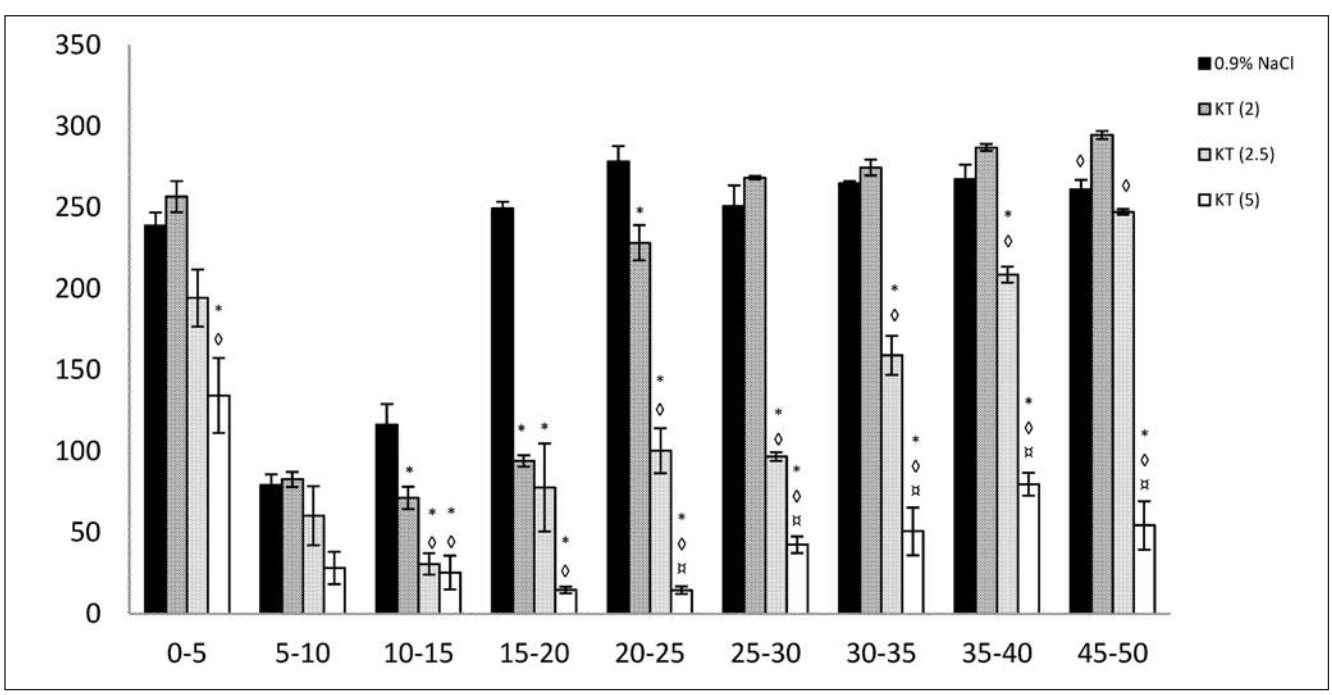

Fig. 2.The antinociceptive effect of ketamine in the formalin test in rats. Each point represents the mean ±SEM of the antinociceptive latency time in seconds (s) obtained in 6–8 rats. At each time interval the differences between the corresponding means were verified using one-way analysis of variance (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); with KT(2) (◊*P*<0.05); with KT (2.5) (¤*P*<0.05).

The effects of the combinations of ketamine-magnesium sulfate and magnesium sulfate-ketamine in the formalin test in rats

Different doses of ketamine (2, 2.5 and 5 mg/kg) and magnesium sulfate (5 mg/kg) were combined and tested (Fig. 3A). Both combinations (ketamine-magnesium sulfate administration and magnesium sulfate administration prior to ketamine administration) produced a significant effect compared to the control (0.9% NaCl) (P<0.05).

The ketamine-magnesium sulfate combination had a dose-dependent effect (Fig. 3A). The effect of

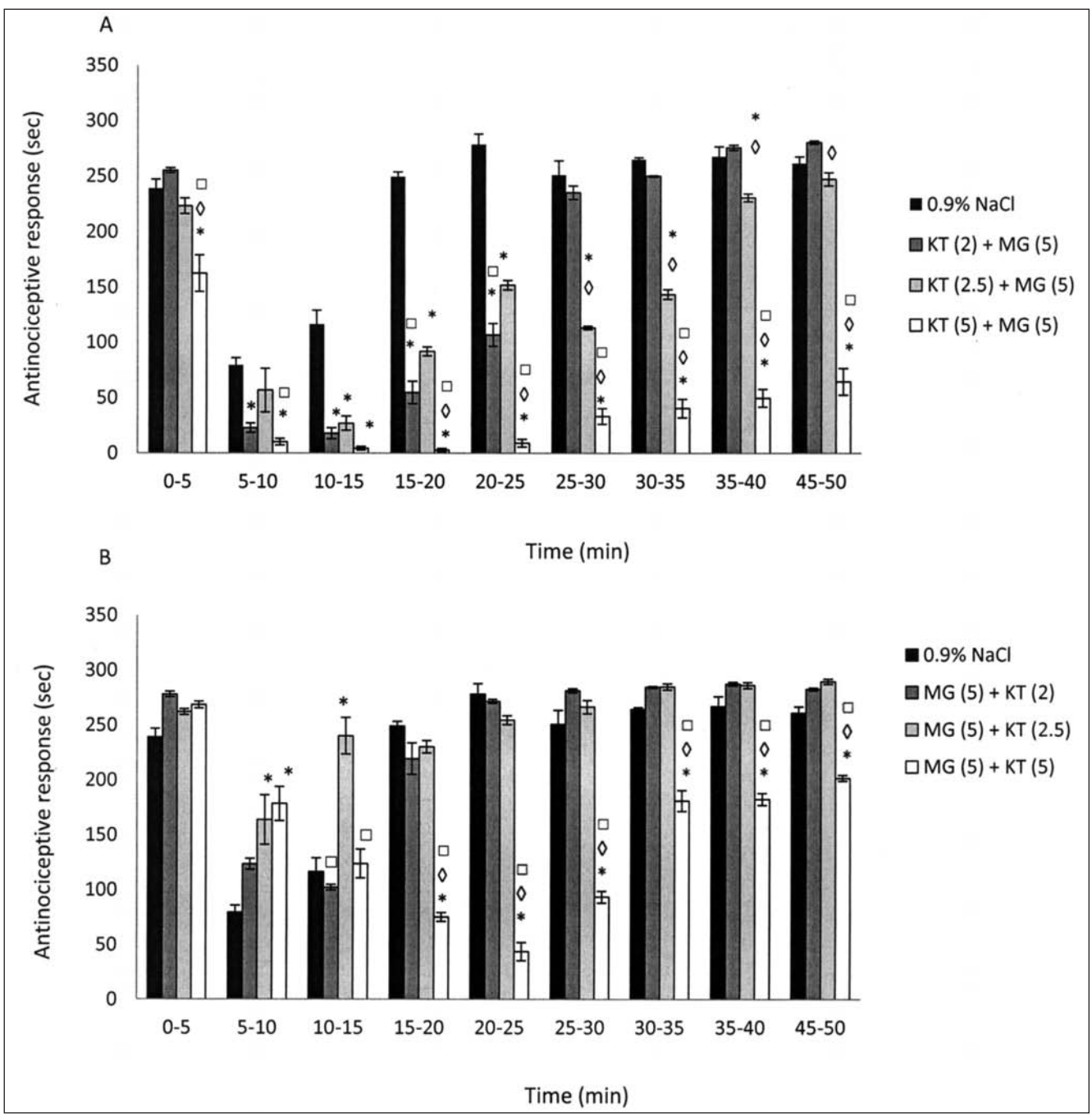

Fig. 3. The antinociceptive effect of the ketamine-magnesium sulfate combination (A) and magnesium sulfate-ketamine combination (B) in the formalin test in rats. Each point represents the mean ±SEM of the antinociceptive latency time in seconds (s) obtained in 6–8 rats. At each time interval the differences between the corresponding means at each time interval 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); with KT(2)+MG(5)/MG(5)+KT(2) ($^{\diamond}P$<0.05); with KT(2.5)+MG(5)/MG(5)+KT(2.5) ($^{\square}P$ <0.05).

the ketamine (2 mg/kg)-magnesium sulfate (5 mg/kg) combination was significantly higher compared to the control (0.9% NaCl) at time points of 5–25 min (P<0.05). The effect of the ketamine (2.5 and 5 mg/kg)-magnesium sulfate (5 mg/kg) combination was significantly higher compared to the control (0.9% NaCl) at time points of 10–45 min (P<0.05). For 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: [$F_{4,25}$=19.597; p=0.000], [$F_{4,25}$=28.838; p=0.000], [$F_{4,25}$=19.950; p=0.000], [$F_{4,25}$=21.083; p=0.000], [$F_{4,25}$=89.298; p=0.000], [$F_{4,25}$=204.658; p=0.000], [$F_{4,25}$=504.817; p=0.000], [$F_{4,25}$=216.012; p=0.000] and [$F_{4,25}$=200.393; p=0.000], respectively.

The magnesium sulfate-ketamine combination did not show a dose-dependent effect (Fig. 3B). The administration of magnesium sulfate (5 mg/kg, sc) followed by ketamine (5 mg/kg, ip) administration after 5 min, produced a significant reduction in formalin-induced pain at the time points from 15–45 min (P<0.05; Fig. 3B). However, magnesium sulfate (5 mg/kg) injected 5 min before the 2 and 2.5 mg/kg doses of ketamine did not affect the nociception (P<0.05; Fig. 3B). For 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: [$F_{3,20}$=11.184; p=0.001], [$F_{3,20}$=5.129; p=0.009], [$F_{3,20}$=17.438; p=0.000], [$F_{3,20}$=95.033; p=0.000], [$F_{3,20}$=96.769; p=0.000], [$F_{3,20}$=125.415; p=0.000], [$F_{3,20}$=88.641; p=0.000], [$F_{3,20}$=39.157; p=0.000] and [$F_{3,20}$=59.436; p=0.000], respectively.

Interactions between ketamine and magnesium sulfate

Additive interaction between ketamine and magnesium sulfate

The interaction between ketamine (the drug that exerted high antinociceptive efficacy and dose-dependent effect in rats) and magnesium sulfate (the drug that exerted low antinociceptive efficacy in rats and a non-dose-dependent effect) was evaluated by co-administration of a fixed, subeffective dose of magnesium sulfate along with increasing doses of ketamine (Gaitan and Herrero 2002). The antinociceptive effects of ketamine were assessed from the corresponding log dose-response curve (ED_{50}=2 mg/kg). In the next step, we examined the combination of a fixed quantity dose of magnesium sulfate (5 mg/kg) along and 3 different doses (2, 2.5 and 5 mg/kg, ip) of ketamine (fixed fractions of the ED_{50}: 1 ED_{50}, 5/4 ED_{50}, 2 ED_{50}). Lower doses of ketamine (1 mg/kg) in combination with magnesium sulfate (5 mg/kg) did not have a significant effect when compared with the control (not shown). The log dose-response curves for ketamine (2, 2.5 and 5 mg/kg, ip) administered alone, and ketamine (2, 2.5 and 5 mg/kg, ip) administered prior to the administration of a fixed dose of magnesium sulfate (5 mg/kg, sc), were constructed and compared (Fig. 4A). There was a non-significant leftward shift of the log dose-response regression curve for ketamine in the presence of magnesium sulfate, compared with the

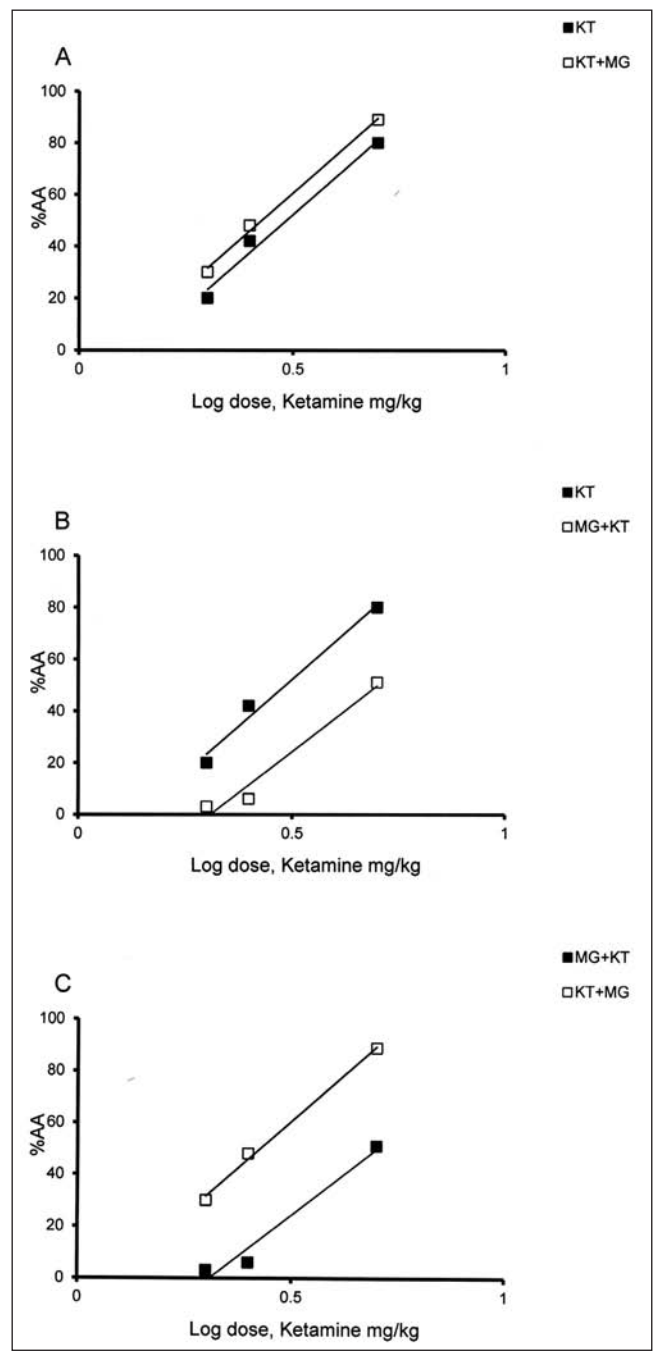

Fig. 4. Log dose-response for (A) ketamine (KT; 2, 2.5 and 5 mg/kg; sc) and the ketamine (2, 2.5 and 5 mg/kg)-magnesium sulfate (5 mg/kg) combination; (B) ketamine (KT; 2, 2.5 and 5 mg/kg; sc) and the magnesium sulfate (5 mg/kg)-ketamine (2, 2.5 and 5 mg/kg) combination; (C) the ketamine (2, 2.5 and 5 mg/kg)-magnesium sulfate (5 mg/kg) and magnesium sulfate (5 mg/kg)-ketamine (2, 2.5 and 5 mg/kg) combinations in the formalin test in rats. Data are expressed as a percent of antinociception − AA (%).

log dose-response regression curve for ketamine alone ($P>0.05$; relative potency test). This points to an additive interaction between ketamine and magnesium sulfate. The potency ratio was 1.2 (CL 0.95–1.38). The slopes are not significantly different ($P<0.05$; test for parallelism).

Antagonistic interaction between ketamine and magnesium sulfate

We compared the curves obtained for ketamine alone and the magnesium sulfate-ketamine combination (Fig. 4B), where ketamine was administered after the magnesium-sulfate. There was a significant rightward shift of the log dose-response regression curve for ketamine in the presence of magnesium sulfate, when compared with the log dose-response regression curve for ketamine alone ($P<0.05$; relative potency test). This points to antagonism between ketamine and magnesium sulfate in the formalin test in rats. The potency ratio was 1.6 (CL 1.2–2.4), confirming an antagonistic interaction. The slopes are not significantly different ($P<0.05$; test for parallelism).

The potency of a ketamine/magnesium sulfate combination is influenced by the order of drug administration

Fig. 4C illustrates the log dose-response curves for ketamine-magnesium sulfate and magnesium sulfate-ketamine combinations. There was a significant rightward shift of the log dose-response regression curve for the magnesium sulfate-ketamine combination compared to the log dose-response regression curve for the ketamine-magnesium sulfate combination, which indicates the importance of the order of drug administration. The slopes are not significantly different ($P<0.05$; test for parallelism). The potency ratio was 1.8 (CL 1.4–2.5).

DISCUSSION

The major findings in the present study are the additive and antagonistic interactions between two NMDA antagonists, ketamine and magnesium sulfate, in the second phase of the formalin test in rats. We combined ketamine, a drug with a high antinociceptive efficacy, and magnesium sulfate, drug with low antinociceptive efficacy. However, the administration of magnesium before or after ketamine resulted in an increase or decrease in the time the animals spent in pain-related behavior, respectively. For the first time, it was demonstrated that in inflammatory pain the order of administration of these medications is important; a lower level of activity was demonstrated when magnesium sulfate was administered before ketamine. Also, this is the first study to show additive and antagonistic interactions between ketamine and magnesium sulfate with statistical confirmation.

In agreement with the findings of the present work, Ishizaki and others (1999) showed that intrathecal magnesium sulfate produced a depression of pain responses in the formalin test only after the first 10 min. The authors concluded that magnesium sulfate did not display remarkable antinociceptive effects in acute pain models. Similar to this, Takano and colleagues (2000) reported that the intrathecal injection of magnesium was capable of decreasing the second phase responses in the formalin test in a dose-dependent manner. However, Begon and others (2002) showed that systemic administration of magnesium sulfate (30 and 90 mg/kg, ip) had no effect on the second phase of the formalin test. On the contrary, it was demonstrated that intraperitoneal magnesium oxide had an antinociceptive effect in both phases of the formalin test, as well as in the writhing test in mice (Jahangiri et al. 2013). Our previous results showed that magnesium sulfate and MK-801 are effective against acetic acid-induced visceral and carrageenan-induced somatic inflammatory pain models in rats (Vuckovic et al. 2015a). Unlike MK-801, the effects of magnesium sulfate were not dose-dependent. In the present experiments magnesium sulfate demonstrated a low antinociceptive activity in the formalin test in rats.

In rodents, the systemic administration of ketamine suppressed pain-related behavior (Sawynok and Reid 2002, Bulutcu et al. 2002, Petrenko et al. 2006, do Vale et al. 2016) and paw swelling (Sawynok and Reid 2002, do Vale et al. 2016) induced by formalin injection into the paw. Ketamine (5–10 mg) reduced the pain response in the second phase (Bulutcu et al. 2002, Petrenko et al. 2006) or in both phases (do Vale et al. 2016) of the formalin test. Also, ketamine at as low a dose as 0.1 mg/kg decreased the time of licking of the inflamed paw in both phases of the formalin test in mice, and co-administration of conventional/nanoparticle magnesium oxide potentiated the effect of ketamine (Jahangiri et al. 2013). In the present experiments magnesium sulfate demonstrated a more pronounced antinociceptive activity in the second phase of the formalin test.

Analysis of the log dose-response curves for the effects of ketamine and the magnesium sulfate-ketamine combination in formalin-induced nociception revealed an antagonistic interaction and a 1.6 (CL 1.2–2.4)-fold increment in ketamine dosage when ketamine was applied after the magnesium sulfate. In addition, a low dose of magnesium sulfate (5 mg/kg, sc) administered after ketamine, increased the antinociceptive effect of ketamine by a factor of only 1.2 (CL 0.95–1.38), pointing to an additive interaction. Therefore, the order of administration of these drugs is most likely important.

There was a 1.8-fold reduction in the ketamine effect when ketamine was administered before rather than after magnesium sulfate. We have previously reported that the efficacy of the ketamine-magnesium sulfate combination in the acute pain (tail immersion) test in rats is influenced by the order of medication administration (Savic Vujovic et al. 2015, Vučković et al. 2015b); a higher level of activity was demonstrated when ketamine was administered before magnesium sulfate (Savic Vujovic et al. 2015, Vučković et al. 2015b). The results of the present study are in agreement with previous ones.

The mechanism that underlies the magnesium and ketamine interaction at the level of the NMDA receptor is not clear. We previously hypothesized that when applied first, the magnesium ions block the NMDA ion channel before ketamine binds to the phencyclidine site, thus reducing its antinociceptive action (Savic Vujovic et al. 2015). Since a synergistic interaction between ketamine and magnesium was previously observed in acute nociceptive pain in rats (Savic Vujovic et al. 2015), we also hypothesized that the type of interaction between ketamine and magnesium depends on the pain model. Acute nociceptive and inflammatory pain are characterized by low and high levels of NMDA receptor activity, respectively (Voscopoulos and Lema 2010). In a previous study we showed that ketamine and magnesium, both NMDA antagonists, are not effective against acute pain when administered alone (Savic Vujovic et al. 2015). However, in this study of inflammatory pain, they are more and less effective, respectively. Ketamine decreases the "wind up" phenomenon, and the antagonism is more important if the NMDA channel has been previously opened by glutamate binding ("use dependence") (Mion and Villevieille 2013, Ziv et al. 2016). This "use dependence" concept can explain why ketamine is more efficient in intense or chronic pain (De Kock et al. 2001) and less efficient in acute pain (Savic Vujovic et al. 2015), or when NMDA receptor activity is lowered by magnesium, as is the case herein.

Further possible explanations for the diverse interactions between ketamine and magnesium may be due to the allosteric modulation of NMDA receptors. The N-terminal domains (NTDs) of GluN2B receptors contain a modulatory site that allows positive allosteric modulation (Mony et al. 2009). This site binds spermine and spermidine, endogenous polyamines, as well as the magnesium ion. A general mechanistic model for allosteric signaling, both positive and negative *via* the NTDs, has been proposed (Lu et al. 1998, Mony et al. 2009, Zhu et al. 2015).

The interaction between ketamine and magnesium described in this study might be due to some other mechanisms that do not involve NMDA receptors. Ketamine increases the release of monoamine neurotransmitters (norepinephrine, dopamine and serotonin) and inhibits their uptake, thus enhancing the descending inhibitory pain pathways (Tso et al. 2004, Koizuka et al. 2005). Ketamine can also interact with nicotinic cholinergic (Scheller et al. 1996, Yamakura et al. 2000), monoaminergic (Kapur and Seeman 2002) and opioid (Sarton et al. 2001, Pacheco Dda et al. 2014) receptors and nitric oxide synthase (do Vale et al. 2016), as well as with hyperpolarisation-activated cyclic nucleotide channels (HCN1) (Chen et al. 2009).

Magnesium also blocks presynaptic and postsynaptic calcium channels, modulates potassium channels and functions through other mechanisms of action (Matsuda et al. 1987, Bara et al. 1993, Mak and Foskett 1998, Shi and Cui 2001, Shi et al. 2002, Shimosawa et al. 2004, Guiet-Bara et al. 2007). In studies using inhibitors of NOS it was recently suggested that the activation of the NO pathway could have an important role in the antinociceptive effects of systemic magnesium sulfate in rats with inflammatory pain (Srebro et al. 2014). It has also been reported that magnesium deficiency produces mechanical hyperalgesia in rats which can be reversed by NMDA receptor antagonists (Dubray et al. 1997, Begon et al. 2001, 2002). Magnesium deficiency induces sensitization of nociceptive pathways which involves NMDA receptors. Oral administration of magnesium can restore thermal hyperalgesia and magnesium deficiency in diabetic rats (Hasanein et al. 2006).

Literature data indicate that both ketamine and magnesium increase opioid antinociception in different animal models of pain (Begon et al. 2002, Alvarez et al. 2003, Savic Vujovic et al. 2015, Bujalska-Zadrożny et al. 2016). In addition, evidence for the involvement of endogenous opioids and mu and delta opioid receptors in ketamine-induced antinociception has been presented (Sarton et al. 2001, Pacheco Dda et al. 2014). Our previous study demonstrated that the morphine-ketamine-magnesium sulfate combination was antagonized by naloxone, an antagonist of opioid receptors, indicating that this interaction is most likely mediated *via* opioid receptors (Vučković et al. 2015b). The NMDA receptor has been shown to associate post-synaptically with mu receptor (Rodrigez-Munoz et al. 2012). When morphine activates the mu receptor, the resulting phosphorylation of a residue of the NMDA receptor causes the dissociation of both receptors and mu receptor desensitization (Rodrigez-Munoz et al. 2012). Like ketamine, magnesium is a NMDA receptor antagonist and can prevent NMDA receptor phosphorylation and opioid-induced hyperalgesia (Bujalska-Zadrożny et al. 2016).

In the second phase of the rat formalin test we observed both additive and antagonistic interactions

between ketamine and magnesium sulfate. The type of interaction depends on the order of administration of these drugs. Similar to our previous results obtained on the model of acute nociceptive pain in rats, higher antinociceptive activity was observed when ketamine was administered before magnesium. The present study also revealed an antagonistic interaction when magnesium sulfate was administered before ketamine. Studies in which a ketamine-magnesium combination was examined in humans provided conflicting results. A double-blind randomized controlled trial group of patients treated during induction of general anesthesia with a combination of magnesium sulfate and S(+)-ketamine showed a trend towards more opioid piritramide use postoperatively via patient controlled analgesia (PCA) device compared with ketamine alone (Stessel at al. 2013), suggesting an antagonistic effect of magnesium on ketamine analgesia. In contrast to this, the combined use of ketamine and magnesium reduced morphine consumption after scoliosis surgery in a prospective randomised double-blind study (Jabbour et al. 2014). However, the order of administration of these drugs in human studies has not been reported.

Since pain often has a mixed etiology, with nociceptive, inflammatory and neuropathic components, it is always advisable to administer ketamine before magnesium in situations when the two drugs are applied together. Further studies are needed to confirm the clinical relevance of the order of ketamine and magnesium sulfate administration in different types of pain (i.e. postoperative, cancer).

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