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Antinociceptive properties of *Micrurus lemniscatus* venom

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ABSTRACT

The therapeutic potential of snake venoms for pain control has been previously demonstrated. In the present study, the antinociceptive effects of *Micrurus lemniscatus* venom (MlV) were investigated in experimental models of pain. The antinociceptive activity of MlV was evaluated using the writhing, formalin, and tail flick tests. Mice motor performance was assessed in the rota rod and open field tests. In a screening test for new antinociceptive substances – the writhing test – oral administration of MlV (19.7–1600 μg/kg) produced significant antinociceptive effect. The venom (1600 μg/kg) also inhibited both phases of the formalin test, confirming the antinociceptive activity. The administration of MlV (1600 μg/kg) did not cause motor impairment in the rota rod and open field tests, which excluded possible non-specific muscle relaxant or sedative effects of the venom. The MlV (177–1600 μg/kg) also increases the tail flick latency response, indicating a central antinociceptive effect for the venom. In this test, the MlV-induced antinociceptive effect was long-lasting and higher than that of morphine, an analgesic considered the gold standard. In another set of experiments, the mechanisms involved in the venom-induced antinociception were investigated through the use of pharmacological antagonists. The MlV (1600 μg/kg) antinociceptive effect was prevented by naloxone (5 mg/kg), a non-selective opioid receptor antagonist, suggesting that this effect is mediated by activation of opioid receptors. In addition, the pre-treatment with the μ-opioid receptor antagonist CTOP (1 mg/kg) blocked the venom antinociceptive effect, while the k-opioid receptor antagonist nor-BNI (0.5 mg/kg) or the δ-opioid receptor antagonist naltrindole (3 mg/kg) only partially reduced the venom-induced antinociception. The present study demonstrates, for the first time, that oral administration of *M. lemniscatus* venom, at doses that did not induce any motor performance alteration, produced potent and long-lasting antinociceptive effect mediated by activation of opioid receptors.

1. Introduction

Physiological pain serves as a warning mechanism that indicates imminent tissue damage. Chronic pain lacks such protective function, since it persists for years without reflecting the severity of a lesion or disease, nor does chronic pain necessarily respond to treatment of the underlying disease cause (McGreevy et al., 2011). Chronic

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pain, despite their enormous morbidity, social cost and damaging effect on quality of life, has limited therapeutic success, which may be due, at least in part, to the small range of useful drugs (O'Connor, 2009). In addition, the pain is still poorly managed because most existing analgesics for persistent pain are relatively ineffective, have a high side effect burden and do not reduce pain in all treated individuals (Woolf, 2010). Therefore, the development of new agents with more powerful analgesic activities and with lesser side effects is, at present, of great interest.

Since ancient times, natural products have consistently been an important source of therapeutic agents. Snake venoms are composed of a wide variety of proteins and peptides, which are used mainly to paralyze prey and as a defense against predators (Kapoor, 2010). Moreover, the property of selectivity of the molecules present in snake venoms, to their molecular targets, makes these molecules useful for the design of novel therapeutic drugs. Several snake venoms and their toxins have analgesic effects already demonstrated in humans and antinociceptive properties in animal models of pain. Hannalgesin, a neurotoxin from the venom of *Ophiophagus hannah*, exhibits antinociceptive activity without causing any neurological or muscular deficits (Pu et al., 1995). The antinociceptive effect of the venom of South American rattle snake, *Crotalus durissus terrificus*, has also been investigated. There is evidence that this effect is due to central mechanisms (Picolo et al., 1998) and mediated by activation of opioid receptors (Giorgi et al., 1993). In addition, the contribution of peripheral mechanisms to that antinociception also has been demonstrated (Picolo et al., 2000, 2003). Crotoxin, the principal neurotoxin in *Crotalus durissus terrificus* venom, has antinociceptive activity in experimental models of pain (Zhang et al., 2006), and reduced pain in patients with advanced cancer (Cura et al., 2002). Crotamine, other neurotoxin from *Crotalus durissus terrificus* venom, is reported to produce antinociceptive effect 30-fold higher than that of morphine without any apparent toxicity (Mancin et al., 1998). In addition, crotalphine, a novel peptide isolated from this venom, induces potent antinociceptive effect mediated by activation of opioid receptors (Konno et al., 2008; Gutierrez et al., 2008). The venom of *Naja naja atra*, a snake of the Elapidae family, presents relevant antinociceptive activity attributed to a venom constituent, the neurotoxin cobrotoxin (Chen and Robinson, 1990; Grasset, 1952; Yang, 1999). Xiong et al. (1992) showed that this toxin might be clinically useful as a substitute for morphine in patients with opioid dependence. Nowadays, cobrotoxin is commercially available for its analgesic effect.

Coral snakes, the representative snakes of the Elapidae family in the Americas, include the genera *Leptomicrurus*, *Micruroides*, and *Micrurus*. The latter is found in regions ranging from the southern of the United States to the southern of South America (Da-Silva and Sites, 2001). Experimental studies suggest the presence of a considerable spectrum of biological activities of *Micrurus* venoms, such as neurotoxic, myotoxic, hemorrhagic, and edematogenic (Cecchini et al., 2005; Francis et al., 1997; Gutiérrez et al., 1992). In addition, many enzymatic activities have been detected (Cecchini et al., 2005). However, due to the difficulty in maintenance in captivity and of the minute quantities of venom obtained from *Micrurus* sp., the pharmacological properties of most of their components remain unknown or poorly understood. The present pharmacological study was undertaken to investigate the antinociceptive property of the *Micrurus lemniscatus* venom (MlV). In addition, the mechanisms of the antinociceptive effect were evaluated.

2. Materials and methods

2.1. Animals

Experiments were performed on male Swiss Webster mice (18–22 g) obtained from the Animal Facilities of Centro de Pesquisas Gonçalo Moniz. Animals were housed in temperature-controlled rooms (22–25 °C), under a 12:12 h light–dark cycle, with access to water and food ad libitum until use. All behavioral tests were performed between 8:00 a.m. and 5:00 p.m., and animals were only used once. Animal care and handling procedures were in accordance with the International Association for the Study of Pain guidelines for the use of animals in pain research (Zimmermann, 1983) and the Institutional Animal Care and Use Committee FIOCRUZ CPqGM 009/2011. Every effort was made to minimize the number of animals used and any discomfort.

2.2. Compounds, dilutions and administration

Dry crude snake venom of *M. lemniscatus* (MlV) was obtained from the Center for the Study of Animal Venom (NEVA), Salvador, Brazil, and stored at −20 °C. The venom, diluted in physiological saline at the time of use, was administered by oral route 1 h before testing. The venom treatment parameters were based on preliminary data from our laboratory. Indomethacin, naloxone (non-selective antagonist of opioid receptors), naltrindole (δ-opioid receptor antagonist), and nor-binaltorphimine (Nor-BNI; κ-opioid receptor antagonist) were purchased from Sigma Chemical Company (St. Louis, MO, USA). D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide (CTOP; μ-opioid receptor antagonist) was purchased from Tocris Bioscience (Bristol, UK). Diazepam and morphine were purchased from Cristália (Itapira, São Paulo, Brazil). Indomethacin was dissolved in Tris HCl 0.1 M pH 8.0 plus physiological saline. Remaining drugs were dissolved in physiological saline. The drugs were administered by oral (p.o.), intraperitoneal (i.p.) or subcutaneous (s.c.) routes. The concentration was adjusted so that all doses could be administered in a fixed volume of 200 μL per animal.

2.3. Nociceptive tests

2.3.1. Writhing test

Acetic acid (0.8% v/v, 10 mL/kg) was injected into the peritoneal cavities of mice, which were placed in a large glass cylinder and the intensity of nociceptive behavior was quantified by counting the total number of writhes occurring between 0 and 30 min after the stimulus injection (Collier et al., 1968).

2.3.2. Formalin test

Mice were placed in an open Plexiglas observation chamber for 10 min in order for them to adapt to their surroundings. Mice were gently restrained while the dorsum of the hind paw was subcutaneously administered with 20 μL of formalin 2.5% (1:100 dilution of stock formalin solution, 37% formaldehyde in 0.9% saline). Following injection, the mice was returned to the observation chamber. Mice were observed from 0 to 10 min (early phase) and from 10 to 30 min (late phase). The nociception score was determined by counting the time that the animal spent licking or biting the injected limb during the observation time (Dubuisson and Dennis, 1977).

2.3.3. Tail flick test

The tail flick test in mice was conducted as described elsewhere (D'Amour and Smith, 1941), with minor modifications. Before the day of the experiment, each animal was habituated to the restraint cylinder for 5 consecutive days (20 min per day). On the experimental day, mice were placed in the restraint cylinder and the tail tip (2 cm) was immersed in a water bath at 48 °C ± 0.5 °C. The latency for the tail withdrawal reflex was measured. Each trial was terminated after 10 s to minimize the probability of skin damage. Tail flick latency was measured before (baseline) and after treatments.

2.4. Assay of motor function

To evaluate possible non-specific muscle-relaxant or sedative effects of *M. lemniscatus* venom, mice were submitted to the rota rod test (Kuribara et al., 1977). The rota rod apparatus (Insight, Ribeirão Preto, Brazil) consisted of a bar with a diameter of 3 cm, subdivided into five compartments. The bar rotated at a constant speed of 6 revolutions per min. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for two consecutive periods of 120 s. Animals were treated with diazepam (10 mg/kg i.p.), venom (1600 μg/kg p.o.), or vehicle (200 μL p.o.), and 40 min afterward, were placed on a rotating rod. The resistance to falling was measured up to 120 s. The results are expressed as the average time (s) the animals remained on the rota rod in each group. To assess the possible effects of *M. lemniscatus* venom on locomotor activity, mice were evaluated in the open-field test (Rodrigues et al., 2002). Mice were treated with diazepam (10 mg/kg i.p.), venom (1600 μg/kg p.o.), or vehicle (200 μL p.o.), and 40 min afterward were placed individually in a wooden box (40 × 60 × 50 cm) with the floor divided into 12 squares. The number of squares crossed with the four paws was measured for a period of 3 min.

2.5. Data analysis

All data are presented as means ± standard error of the mean (S.E.M) of measurements made on six animals in each group. All data were analyzed using the Prism 5 computer software (GraphPad, San Diego, USA). Comparisons across three or more treatments were made using one-way ANOVA with Tukey's post hoc test or repeated measures two-way ANOVA with Bonferroni's post hoc test, when appropriate. Statistical differences were considered to be significant at $p < 0.05$. For the dose–response analysis, the data of tail flick latencies were converted to antinociception index (%), according to the formula:

$$AI = \frac{(test\ latency - baseline\ latency)}{cut - off\ time(10\ s) - baseline\ latency} \times 100$$

3. Results and discussion

The antinociceptive properties of *M. lemniscatus* venom were evaluated initially using the writhing test in mice, a screening tool for the assessment of antinociceptive properties of new substances (Collier et al., 1968). Preliminary data from our laboratory showed that the oral administration of *M. lemniscatus* venom presents improved antinociceptive effect in relation to the intraperitoneal administration (data not shown). So, in the present study the oral route was used to further characterization of the antinociceptive properties of *M. lemniscatus* venom.

Oral administration of MlV (19.7–1600 μg/kg), 1 h before acetic acid injection, produced a significant ($p < 0.05$) inhibition of acetic acid-induced abdominal constrictions in mice (Fig. 1). Indomethacin (10 mg/kg i.p.), a standard NSAID used as a positive control, 30 min before testing also produced a significant inhibition of the acetic acid-induced writhing response. The writhing test presents a good sensitivity, although with poor specificity. Indeed, this test works not only for analgesics, but also for several other substances, including some devoid of antinociceptive action, e.g., adrenergic blockers, muscle relaxants, and neuroleptics (Le Bars et al., 2001). Thus, a positive result with this test does not necessarily mean the presence of antinociceptive activity. To avoid misinterpretation of the results, we confirmed the antinociceptive effect of MlV using the formalin test, which has two distinct phases that can possibly indicate different types of pain (Hunskaar and Hole, 1987). The early and late phases of the formalin test have clearly different properties, and therefore it is useful not only to assess antinociceptive substances but also for the elucidation of the mechanisms of antinociception (Shibata et al., 1989). The early phase, named nociceptive, results essentially from the direct stimulation of nociceptors, whereas the late phase, named inflammatory, involves a period of central and peripheral sensitization during which inflammatory phenomena occur (Hunskaar and Hole, 1987). Injection of formalin in control animals induced a biphasic flinching response, with the early phase ranging from 0 to 10 min (Fig. 2A) and the late phase from 10 to 30 min (Fig. 2B) after the injection. Treatment with MlV (1600 μg/kg) by oral route 1 h before the formalin administration caused an antinociceptive effect ($p < 0.05$) in both the early and late phases of formalin test. The results obtained with control groups support the antinociceptive effects of *M. lemniscatus* venom, since the saline had no activity, and the standard drug morphine (5 mg/kg s.c.) also inhibited formalin-induced nociception. Moreover, relaxing or motor deficit effects were discarded, since administration of *M. lemniscatus* venom at therapeutic doses (1600 μg/kg) did not affect the motor

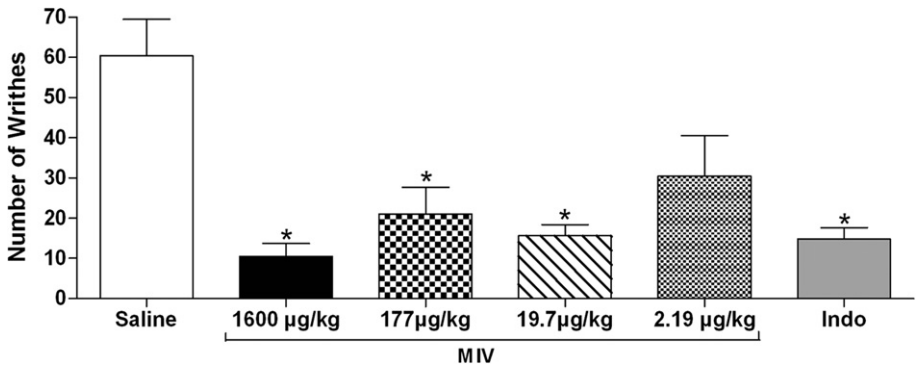

Fig. 1. Effects of oral administration of *M. lemniscatus* venom (MlV) on acetic acid-induced writhing in mice. Mice were treated with MlV (2.19–1600 µg/kg) or saline (200 µL; control group) by oral route 1 h before acetic acid 0.8% (injected at time zero). Indomethacin (Indo; 10 mg/kg i.p.), the reference drug, was administered 30 min before the acid injection. Data are expressed as means ± S.E.M.; $n = 6$ mice per group. *Significantly different from control group ($p < 0.05$) as determined by ANOVA followed by Tukey's test.

performance of the mice, as tested in the rota rod (Fig. 3A) and in the open field (Fig. 3B) tests. As expected, the central nervous system depressant diazepam (10 mg/kg i.p.) reduced the time of mice on the rota rod (Fig. 3A) and the number of crossings on the open field (Fig. 3B) after 30 min of treatment with this standard drug ($p < 0.001$). This result indicates that the effect of the *M. lemniscatus* venom observed in the nociceptive models does not result from alterations in the locomotor activity of the animals, confirming that this venom induces antinociceptive effect. In line with the present results, it was demonstrated that neurotoxins from snake venoms present antinociceptive activity without causing neurological or motor deficits (Mancin et al., 1998; Pu et al., 1995).

Nonsteroidal anti-inflammatory drugs seem to suppress only the second phase of formalin test. In contrast, central

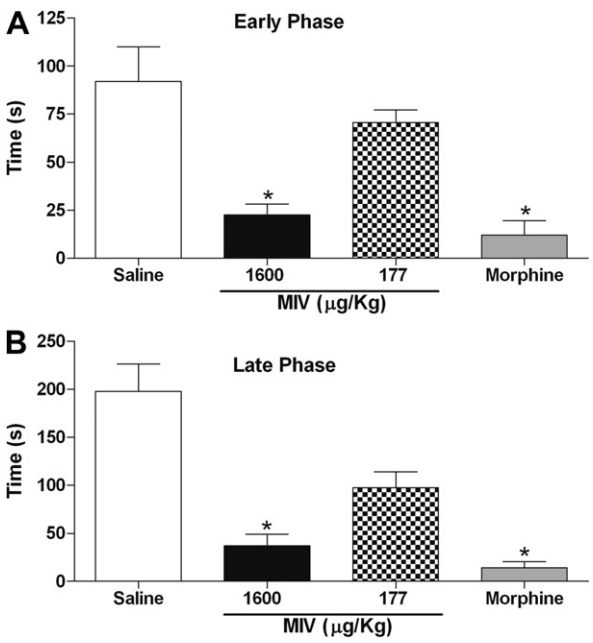

Fig. 2. Effects of oral administration of *M. lemniscatus* venom (MlV) on formalin test in mice. Panels A and B show the effects of MlV on the early and late phases of formalin-induced flinches, respectively. Mice were treated with MlV (177 and 1600 µg/kg) or saline (200 µL; control group) by oral route 1 h before formalin (injected at time zero). Morphine (5 mg/kg s.c.), the reference drug, was administered 40 min before the formalin injection. Data are expressed as means ± S.E.M.; $n = 6$ mice per group. *Significantly different from control group ($p < 0.05$) as determined by ANOVA followed by Tukey's test.

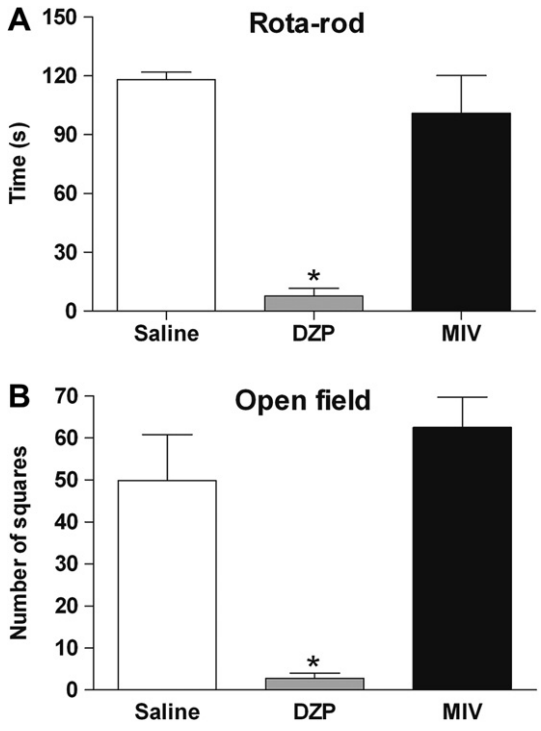

Fig. 3. Effects of *M. lemniscatus* venom (MlV) on motor function. Bar graphs representing (A) the run time on the rota rod and (B) the number of square crossings in the open field test, 1 h after the oral administration of MlV (1600 µg/kg) or saline (200 µL; control group). Diazepam (DZP; 10 mg/kg), the reference drug, was administered 30 min before testing. Data are reported as means ± SEM; $n = 6$ mice per group. *Significantly different from the control group ($p < 0.001$) as determined by ANOVA followed by the Tukey's test.

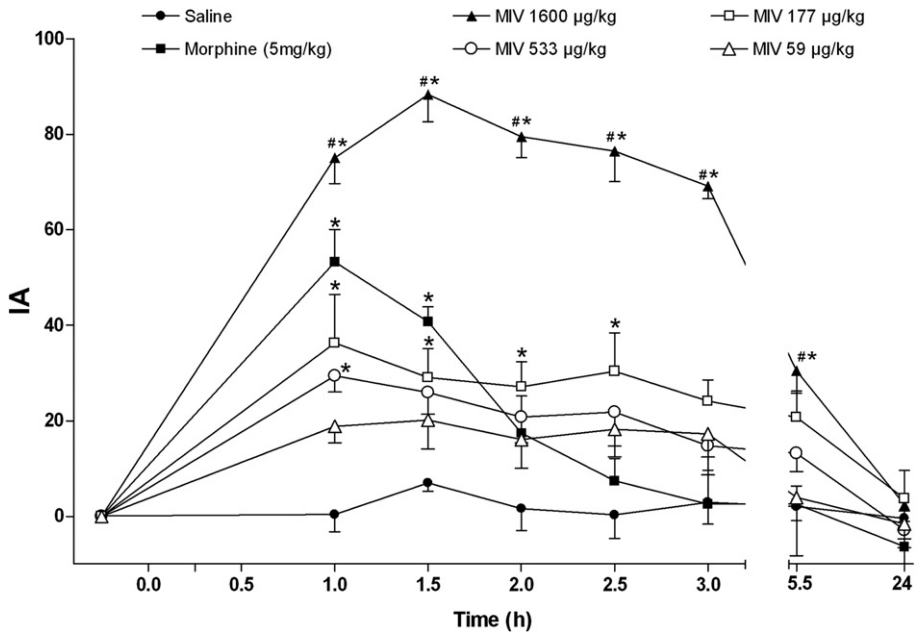

Fig. 4. Characterization of the antinociceptive effect of *M. lemniscatus* venom (MlV) in the tail flick test. The figure show data of tail flick latencies represented as antinociception index (IA). For the dose–response analysis, the effects of increasing doses of MlV (59–1600 µg/kg) were tested. To evaluate the time-course of the antinociceptive effect, thermal nociceptive threshold was evaluated before and up to 24 h following oral administration of MlV or saline (200 µL; control group). Morphine (5 mg/kg s.c.), the reference drug, was administered 40 min before the tail flick test. Data are expressed as means ± S.E.M.; $n = 6$ mice per group. *Significantly different from control group ($p < 0.05$); # significantly different from morphine-treated mice group ($p < 0.05$). Two-way ANOVA followed by the Bonferroni's test.

analgesics, such as opioids, seem to be antinociceptive for both phases (Hunskaar and Hole, 1987; Malmberg and Yaksh, 1992). Considering the inhibitory property of MlV in both the early and late phases of formalin test, it may be suggested that its antinociceptive activity is due, at least in part, to central mechanisms. In fact, snake venoms may induce antinociceptive effects associated with central actions (Giorgi et al., 1993; Picolo et al., 1998). In an attempt to investigate this hypothesis, the effects of treatment with *M. lemniscatus* venom were assessed in the tail flick test,

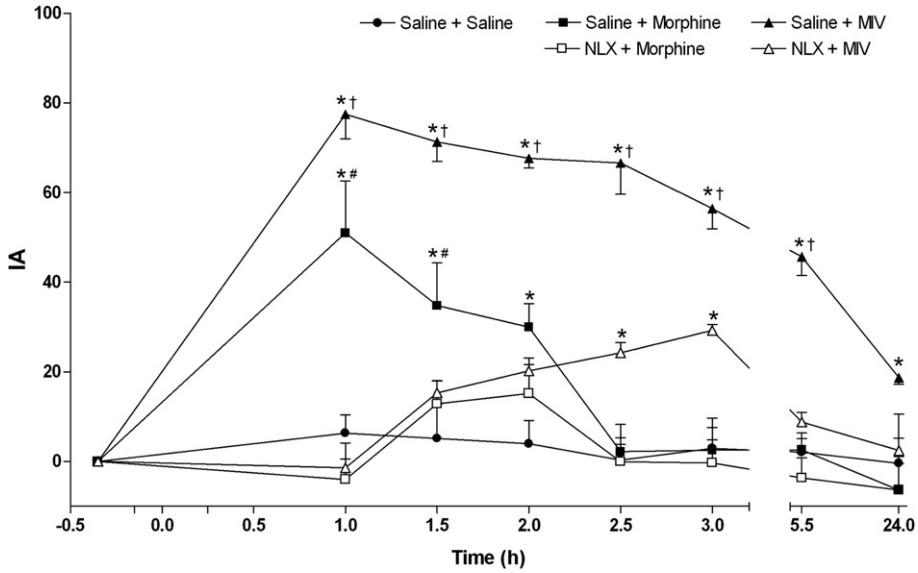

Fig. 5. Effects of the pharmacological blocked of opioid receptors on the antinociceptive effect of *M. lemniscatus* venom (MlV). The figure shows data of tail flick latencies represented as antinociception index (IA). The thermal nociceptive threshold was evaluated before and up to 24 h following oral administration of MlV (1600 µg/kg) or saline (200 µL; control group). Morphine (5 mg/kg s.c.), the reference drug, was administered 40 min before the tail flick test. Naloxone (NLX; 5 mg/kg i.p.), a non-selective opioid receptor antagonist, was administered 15 min before the administration of MlV, morphine or saline. Data are expressed as means ± S.E.M.; $n = 6$ mice per group. *Significantly different from control group ($p < 0.05$); #significantly different from NLX + Morphine group ($p < 0.05$); †significantly different from NLX + MlV group ($p < 0.05$). Two-way ANOVA followed by the Bonferroni's test.

which identifies mainly central analgesics (Le Bars et al., 2001). The oral administration of the venom (177–1600 μg/kg) enhanced the reaction time in the tail-flick test (Fig. 4; $p < 0.05$), an effect that lasted 5.5 h. The administration of morphine (5 mg/kg s.c.), the reference drug, 40 min before testing caused a significant increase in the latency response just 1 h after administration ($p < 0.05$). In addition, the antinociception of the MlV-treated group was significantly higher ($p < 0.05$) relative to the morphine-treated group. The data presented in Fig. 4 show that the antinociceptive effect of the venom was long-lasting and higher than that of morphine, an effect that is hardly reached by analgesics clinically available. In fact, neurotoxins from venoms usually have high pharmacological potency. For instance, the antinociceptive effect of crotamine from *Crotalus durissus terrificus* venom is 30-fold higher than that of morphine (Mancin et al., 1998). This useful property is probably due to the high affinity and selectivity with which these toxins interfere with neuronal mechanisms (Beleboni et al., 2004; Mellor and Usherwood, 2004; Wang and Chi, 2004). The thermal model of the tail flick test is considered to be a spinal reflex, but could also involve higher neural structures (Jensen and Yaksh, 1986; Le Bars et al., 2001). These characteristics of this model are helpful tools to investigate the site of action of antinociceptive agents. On the other hand, antinociceptive activity of opioids with restricted access to the central nervous system was demonstrated in the tail flick test (Fürst et al., 2005), indicating that this test is also sensitive to peripheral acting opioids. In line with this idea, it is possible that *M. lemniscatus* venom exerts its antinociceptive effect both by central and peripheral mechanisms.

The fact that *M. lemniscatus* venom produced antinociception in the tail flick test suggests that it blocks the neural transmission of pain, like opioids do. Based on this possibility, the effects of the pharmacological blocked of opioid receptors on the antinociceptive activity of *M. lemniscatus* venom was evaluated. The maximal antinociception produced by MlV (1600 μg/kg) was completely prevented in mice pre-treated with naloxone (5 mg/kg i.p.; 15 min before), a non-selective opioid receptor antagonist (Fig. 5). The inhibitory effect of naloxone was maintained for 2 h, in line with literature data showing the naloxone half-life (Ngai et al., 1976). The demonstration that naloxone antagonizes the MlV-induced antinociception suggests an opioid-like activity for the venom. Similarly, administration of the μ-opioid receptor antagonist CTOP (1 mg/kg i.p.) 30 min after the MlV administration, blocked the antinociceptive effect of venom (Fig. 6A). On the other hand, the pre-treatment with the k-opioid receptor antagonist nor-BNI (0.5 mg/kg s.c.; 15 min before) partially inhibited the venom-induced antinociception (Fig. 6B). The pre-treatment with naltrindole (3.0 mg/kg s.c.; 5 min before), a δ-opioid receptor antagonist, also reduced the venom-induced antinociception (Fig. 6C). These results suggest that opioid receptors, particularly μ-opioid receptors, play a major role in the antinociceptive mechanisms of MlV. This idea is reinforced by literature data showing that opioid receptors are frequently involved in the antinociceptive effects of snake venoms (Chen et al., 2006; Giorgi et al., 1993; Picolo et al., 2000; Pu et al., 1995).

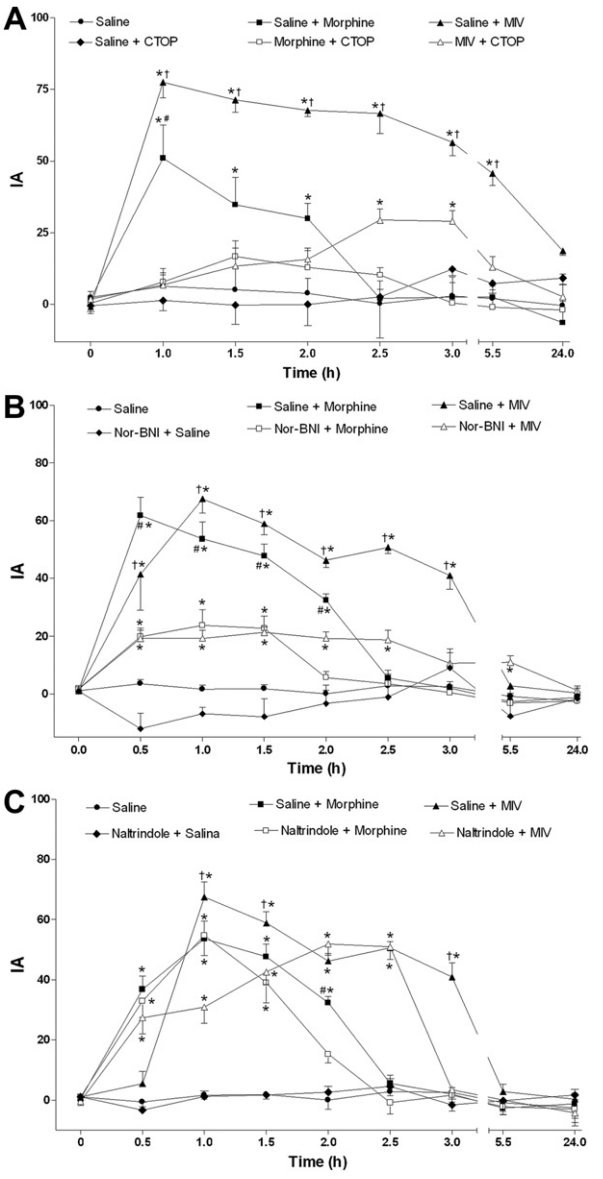

Fig. 6. Effects of selective opioid receptor antagonists on the antinociceptive effect of *M. lemniscatus* venom (MlV). The figure show data of tail flick latencies represented as antinociception index (IA). Thermal nociceptive threshold was evaluated before and up to 24 h following oral administration of MlV (1600 μg/kg) or saline (200 μL; control group). Morphine (5 mg/kg s.c.), the reference drug, was administered 40 min before the tail flick test. Panel A shows the effects of μ-opioid receptor antagonist (CTOP; 1 mg/kg i.p.) on the antinociceptive effect of MlV. CTOP was administered 30 min after the administration of MlV, morphine or saline. Panel B shows the effects of k-opioid receptor antagonist (nor-BNI; 0.5 mg/kg s.c.) on the antinociceptive effect of MlV. Nor-BNI was administered 15 min before the administration of MlV, morphine or saline. Panel C shows the effects of δ-opioid receptor antagonist (naltrindole; 3.0 mg/kg s.c.) on the antinociceptive effect of MlV. Naltrindole was administered 5 min before the administration of MlV, morphine or saline. Data are expressed as means ± S.E.M.; $n = 6$ mice per group. *Significantly different from control group ($p < 0.05$); #significantly different from Antagonist + Morphine group ($p < 0.05$); †significantly different from Antagonist + MlV group ($p < 0.05$). Two-way ANOVA followed by the Bonferroni's test.

4. Conclusions

In conclusion, the present study has demonstrated, for the first time, that oral administration of *M. lemniscatus* venom, at doses that did not induce any apparent toxicity or motor performance alterations, produced potent antinociceptive effects. The antinociceptive effect due to *M. lemniscatus* venom is mediated by the opioid system, mainly by the μ-opioid receptor. However, a more in-depth evaluation of the mechanisms involved should be performed.

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Ethical statement

The authors guarantee that the works presented in this manuscript follow the rules of ethics and respect the duties of authors presented in the Elsevier's Ethical Guidelines for Journal Publication. Experimental procedures were approved by the Institutional Animal Care and Use Committee from FIOCRUZ/CPqGM, Brazil.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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