Journal of
VENOM RESEARCH
ISSN: 2044-0324 J Venom Res, 2022, Vol 12, 1-8
RESEARCH ARTICLE

Analgesic effects of *Naja kaouthia* snake venom and its fractions on inflammatory pain are mediated by peripheral opioid receptors

Joyce T Da Silva[1,2,3X], Milena F Freitas[1X], Vanessa O Zambelli[9], Vanessa P Gutierrez[9], Renata Giorgi[4], Simone Flight[5], Martin Lavin[6], Michael Venning[7], Peter Mirtschin[8], Marucia Chacur[1*]

[1]Department of Anatomy, Institute of Biomedical Sciences, University of São Paulo, Sao Paulo, Brazil

[2]Department of Neural and Pain Sciences, School of Dentistry, Center to Advance Chronic Pain Research, University of Maryland Baltimore, Baltimore, USA

[3]Department of Psychiatry, School of Medicine, Johns Hopkins University, Baltimore, USA

[4]Laboratory of Pathophysiology, Butantan Institute, São Paulo, Brazil

[5]BioPharma Regulatory Consulting, Queensland, Australia

[6]UQCCR, University of Queensland, Brisbane 4029, Australia

[7]School of Pharmacy and Medical Sciences, University of South Australia

[8]Venom Supplies Pty Ltd, Clayton Bay South Australia 5256.

[9]Laboratory of Pain and Signaling, Butantan Institute, Sao Paulo, Brazil

[X]The authors share first authorship.

***Correspondence to:** Marucia Chacur, E-mail: chacurm@icb.usp.br

Received: 30 Sep 2021 | **Revised:** 09 Nov 2021 | **Accepted:** 02 Dec 2021 | **Published:** 06 Jan 2022

ABSTRACT

Venom of cobras of genus *Naja*, including *Naja kaouthia*, can relieve pain in acute and chronic conditions. We investigated the effects of oral and intraplantar administration of the *Naja kaouthia* venom and its fractions on pain-related responses in an inflammatory pain model in rats. Male Wistar rats received a hind paw injection of prostaglandin E2 (PGE2) to induce inflammatory pain and either oral or intraplantar administration of *Naja kaouthia* venom and its fractions (fractions 1 to 5). In addition, separate groups of rats with oral administration of fraction 3 of the *Naja kaouthia* venom also received either μ-, κ- or δ-opioid receptor antagonists, which were injected into the hind paw by intraplantar route. Mechanical thresholds were assessed on the hind paw before and after treatments. Fractionation of *Naja kaouthia* venom was performed using size exclusion chromatography. *Naja kaouthia* venom reduced pain-related responses in the inflammatory pain model when administered by oral and intraplantar routes. Fractions 1, 3, 4 and 5 of the *Naja kaouthia* venom administered by oral route decreased PGE2-induced pain sensitivity, while fraction 2 did not modify pain-related responses. Hind paw injection of naloxone, a non-specific opioid receptor antagonist, abolished the analgesic effects of the *Naja kaouthia* venom as well of that for fraction 3. Additionally, hind paw injection of either μ-, κ- or δ-opioid receptor antagonists blocked the pain relief induced by fraction 3. This study indicates that the *Naja kaouthia* venom and its fractionated forms, particularly fraction 3, may be potential therapeutic targets for pain management and peripheral opioid receptors mediate the pain relief induced by fraction 3.

KEYWORDS: *Naja kaouthia*, thai cobra, snake venoms, inflammatory pain, opioid receptors

INTRODUCTION

Snake venoms comprise a combination of biological active components that are utilized in the development of new drugs to treat many diseases, including Captopril® (Enalapril), Integrilin® (Eptifibatide) and Aggrastat® (Tirofiban) (Munawar et al, 2018; Mohamed Abd El-Aziz et al, 2019). Several drugs in use or in clinical trials have been isolated or derived from snake venom proteins in the past few decades (Mohamed Abd El-Aziz et al, 2019). Additionally, snake venoms have been used to treat joint pain, cancer pain, inflammation and arthritis in complementary and alternative medicine approaches for decades (Gomes et al, 2010; Mohamed Abd El-Aziz et al, 2019; Metzger 2021). For example, the results of the randomized, double-blind, cross-over study have shown that cobrotoxin from the *Ophiophagus hannah* venom may be a valuable therapy for the treatment of chronic moderate to severe pain in cancer patients (Xu et al, 2006). However, the biological bases of the analgesic effects induced by snake venoms remain poorly investigated, particularly involving the venom of the Thai cobra *Naja kaouthia* and opioidergic mechanisms.

Reports on the analgesic actions of snake venoms date from the 1930s and describe the use of *Naja tripudians* and *Crotalus durissus terrificus* venoms in different painful conditions in humans (Monaelesser F and C 1933; Hawgood 1992). Our group has shown that crotoxin and crotalphine (structural analogue) from the venom of the South American rattlesnake *Crotalus durissus terrificus* administered by oral route has a long-lasting analgesic effect on neuropathic pain model in rats, which is mediated by peripheral opioid receptors, central muscarinic receptors and 5-lipoxygenase-derived mediators (Gutierrez et al, 2008; Nogueira-Neto Fde et al, 2008). Venoms from *Naja* cobras, such as *Naja kaouthia*, *Naja naja atra* and *Naja haje*, can also be used as pain-relief therapies for cancer, neuropathic pain and arthritis (Xu et al, 2006; Gong et al, 2015; Liang et al, 2015; Metzger 2021).

Venom of the Thai cobra *Naja kaouthia* contains several pharmacologically active components, most of which are proteins and polypeptides (Kulkeaw et al, 2007). The signs and symptoms of the *Naja kaouthia* bite have been investigated (Wongtongkam et al, 2005; Gomes et al, 2010). However, the opioidergic mechanisms underlying the analgesic effects of the *Naja kaouthia* venom are still unknown. The pain relief induced by the *Naja kaouthia* venom has been shown in a neuropathic pain model and acetic acid-induced writhing responses using intrathecal, intraperitoneal and brain injections of the α-neurotoxin isolated from the Thai cobra (Chen et al, 2006; Gong et al, 2015). The α-neurotoxin reduced pain-related responses and inhibited thalamic activation. These effects were blocked by atropine and selective α7 nicotinic receptor antagonist, which suggest that the central cholinergic system appears to be involved in the antinociceptive action of the *Naja kaouthia* venom (Chen et al, 2006; Gong et al, 2015). Thus, while the cholinergic effects of the *Naja kaouthia* venom-induced analgesia have been investigated, the opioidergic effects remain unknown.

In this study, we sought to determine the effects of oral and intraplantar administration of *Naja kaouthia* venom and its fractions on an inflammatory pain model induced by hind paw injection of prostaglandin E_2 in rats. We then examined the involvement of peripheral opioid receptors in the antinociceptive effect of oral administration of fraction 3 of the *Naja kaouthia* venom. We hypothesized that mechanical pain-related responses are reduced by *Naja kaouthia* venom and its fractions in an inflammatory pain model and oral administration of fraction 3 may be a potential candidate for the management of painful conditions by promoting modulation of peripheral opioid receptors.

MATERIAL AND METHODS

Animals

Male Wistar rats weighing 180-200g were used in all experiments. Animals were housed in a temperature-controlled (21 ± 2°C) and light-controlled (12:12 light-dark cycle) room with access to food and water *ad libitum* until 2 hours before treatments. After this period, only water was available to the rats. All experiments were performed at the Butantan Institute (Sao Paulo, Brazil) in 2005 and approved by the Institutional Animal Care Committee of the Butantan Institute (CEUAIB, protocol number 248/2005). Efforts were made to minimize the number of animals used and their suffering and sample size was determined based on our previous studies (Gutierrez et al, 2008) (n=5 rats per group for all assays).

Naja kaouthia venom

Lyophilized venom of *Naja kaouthia* was obtained from the Venom Supplies Pty Ltd; Tanunda, Australia, and stored at -20 °C. The venom was diluted in sterile saline and administered by oral (P.O. - gastral cannula) or intraplantar (i.pl.) routes immediately before PGE2 injection. Rats were administered by P.O. with either 200 µL of sterile saline solution (0.15 M NaCl - control group) or 200 µL of saline containing 5, 10, 20 or 40 µg of the *Naja kaouthia* venom (Nk). The venom was also administered by i.pl. with 100 µL of sterile saline solution (control group) or 100 µL of saline containing 0.5, 5 or 10 µg of Nk. Methods and dosage were employed according to our previous studies (Giorgi et al, 1993; Picolo et al, 2000).

Size exclusion chromatography

Fractionation of *Naja kaouthia* venom was performed with size exclusion chromatography using a Sephacryl 300 column (5.0 x 90 cm) (Jose Chirayil et al, 2017). Freeze dried venom was reconstituted to 27 mg/mL in 50 mM Tris-HCl containing 50% glycerol, pH 7.4. Twenty milliliters (540 mg) was loaded onto the Sephacryl 300 column. Venom was eluted with 50 mM Tris-HCl, pH 7.4 at 4^{o}C with detection at 214 nm to detect venom peptide bonds and 280 nm to detect aromatic structures. A total of 115 fractions (8.8 mL per fraction) were collected. The resulting chromatogram identified five peaks (Figure 1). The fractions within each of the five peaks were concentrated using an Ultracel ® YM-3 membrane (Millipore) with a solution exchange to PBS. Fractions were then dried down using a centrifugal evaporator and reconstituted in 0.9M NaCl immediately before use. Rats were administered by P.O. with either 200 µL of sterile saline solution (control group) or 200 µL of saline containing 5, 10 or 20 µg of all five fractions.

Induction of hyperalgesia using prostaglandin E_2

Hyperalgesia was induced in rats by i.pl. of 100 µl of sterile saline containing prostaglandin E_2 (PGE2, 100 ng/paw, Sigma Chem. Co., USA) into the right hind paw as previously described (Picolo et al, 2003; Konno et al, 2008).

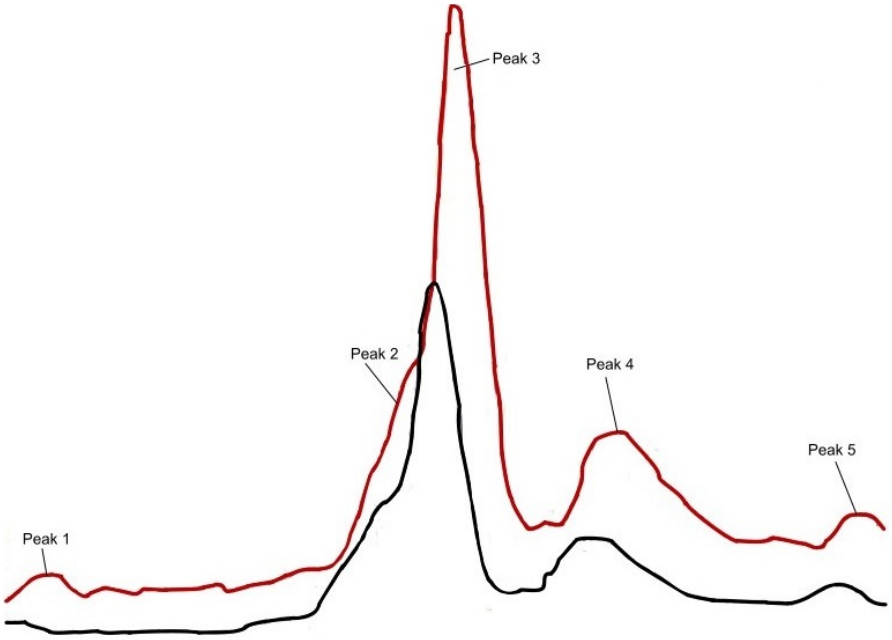

Figure 1: Fractionation of *Naja Kaouthia* snake venom by size exclusion chromatography. The red trace is 280nm and blue is 214nm.

Pharmacological treatments

Nk and fraction 3 (F3) were dissolved in sterile saline and administered by P.O. before intraplantar injection of PGE2 into the right hind paw. To characterize the involvement of opioid receptors in the antinociceptive effect of Nk and F3, naloxone, a non-specific opioid receptor antagonist, was injected by the intraplantar route (1 µg/right hind paw) after oral administration of Nk or F3 and 15 min before mechanical testing. To characterize the type of opioid receptors involved in the antinociceptive effect, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide (CTOP, 20 µg/paw), nor-binaltorphimine (nor-BNI, 50 µg/paw) or N,N-diallyl-Tyr-Aib-Aib-Phe-Leu (ICI 164,864, 10 µg/paw), which are µ-, κ- and δ-opioid receptor antagonists respectively, were injected into the right hind paw by intraplantar route after oral administration of Nk or F3 and 15 min before mechanical testing. The doses of the antagonists were based on previous publications from our group (Picolo et al, 2000; Picolo et al, 2003; Picolo and Cury 2004; Konno et al, 2008). Naloxone was acquired from Rhodia do Brasil. CTOP, nor-BNI, ICI 164,864 were purchased from RBI, USA. PGE2 was purchased from Sigma, USA. Investigators conducting all assays were blinded to the experimental groups and drugs.

Mechanical withdrawal threshold (Randall and Selitto test)

Ugo-Basile pressure apparatus was used to assess mechanical sensitivity by measuring paw withdrawal latency (Randall and Selitto 1957). Increasing pressure (16 g/s) was applied to the right hind paw. The force needed to induce paw withdrawal was recorded as the pain-like threshold. To reduce stress, rats were habituated to the apparatus the day before the experiment (Deuis et al, 2017). The Randall and Selitto test was performed before the injection of PGE2 and 3 hours after treatments (Kayser, 2013). Investigators conducting the behavioral study were blinded to the experimental groups and drugs.

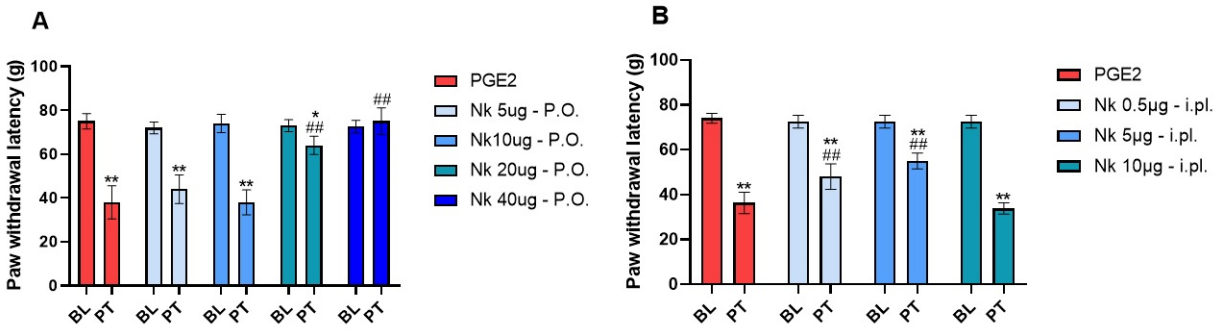

Figure 2: Antinociceptive effect of *Naja kaouthia* venom on mechanical hyperalgesia induced by PGE2. Paw withdrawal latency was assessed before (baseline - BL) and 3 hours after intraplantar injection (post treatment - PT) of PGE2. Nk was administered by oral (A) and intraplantar (B) routes immediately before intraplantar injection of PGE2. Data were presented as the mean ± SEM (n=5 rats per group). Graph A: *p<0.05 and **p<0.0001 compared to within-subject BL, and ##p<0.0001 compared to PGE2 PT. Graph B: **p<0.0001 compared to within-subject BL, and ##p<0.0001 compared to PGE2 PT. BL: baseline, i.pl.: intraplantar route, Nk: *Naja kaouthia venom*, PGE2: prostaglandin E2, P.O.: oral route, and PT: post treatment.

Data analysis
Prism 8 (GraphPad Software Inc., CA, USA) was used to perform statistical analysis. Behavioral measures were analyzed using two-way ANOVA with Bonferroni post-hoc test to determine significant treatment and time effects for each group. Differences were considered statistically significant at $p < 0.05$ and the data were presented as mean ± standard error of the mean (S.E.M.).

RESULTS

Antinociceptive effect of *Naja kaouthia* venom on mechanical hyperalgesia induced by intraplantar injection of PGE2
The intraplantar injection of PGE2 induced a significant decrease in mechanical threshold of the ipsilateral hind paw, characterizing the phenomenon of hyperalgesia (Figure 2). The peak of the hyperalgesic response was observed 3 hours after PGE2 injection. The mechanical threshold of the contralateral hind paw was not changed after PGE2 injection compared to baseline (before treatment: 75 ± 3.28 g; after treatment: 70 ± 2.75 g – data not shown) and intraplantar injection of saline did not modify pain-like thresholds of rats (data not shown).

The doses of 20 and 40 µg of the Nk administered by oral route decreased PGE2-induced mechanical hyperalgesia whereas the lower doses of 5 and 10 µg did not change pain-like thresholds (Figure 2A). The dose of 40 µg (p.o.) was chosen for subsequent experiments since there was no statistical difference between baseline and post-treatment mechanical thresholds. The antinociceptive effect of Nk was also observed when the venom was administered by the intraplantar route (Figure 2B). A dose of 0.5 µg and 5 µg/hind paw decreased PGE2-induced hyperalgesia whereas the higher dose of 10 µg did not change pain-like thresholds (Figure 2B).

Antinociceptive effects of fractions 1 to 5 isolated from *Naja kaouthia* venom on mechanical hyperalgesia induced by intraplantar injection of PGE2
Since *Naja Kaouthia* venom was able to decrease pain sensitivity induced by PGE2, our next step was to observe if the fraction obtained from the crude venom were also able to interfere with pain sensitivity. Sephacryl fractions 1, 3, 4 and 5 (20 µg/kg) administered by oral route decreased PGE2-induced hyperalgesia (Figure 3). The oral administration of fraction 2 (F2) did not modify pain-like thresholds (Figure 3). The intensity of the antinociception observed from F1, 3, 4 and 5 was not statistically different from each other. Based on these results and the purification experiments showing that F3 was the most abundant fraction, F3 was employed in the following experiments investigating potential mechanisms associated with analgesia. Thus, the dose-response curve seen in figure 4 was done via an oral route using F3 only. Figure 4 shows that the antinociceptive effects of 5, 10 and 20 µg doses of F3 were not statistically different from each other. The lower dose of 5 µg of F3 was chosen for subsequent experiments.

Involvement of opioid receptors in the antinociceptive effects of *Naja kaouthia* venom and fraction 3
Rats were injected with naloxone by intraplantar route in order to evaluate the involvement of opioid receptors in the antinociceptive mechanisms induced by Nk and fraction 3. PGE2 was injected in the same paw that received naloxone (1 µg/paw) injection. The antinociceptive effects of Nk (40 µg, P.O.) and F3 (5 µg, P.O.) were abolished in the hind paw injected with naloxone and PGE2 (Figure 5A and 5B).

Since the antinociceptive effect of both Nk and its F3 was abolished by naloxone, we decided to further investigate whether antinociception induced by F3 only (5 µg, P.O.) could be mediated by specific opioid receptors (Figure 6). The administration of CTOP (20 µg/paw), NOR (50 µg/paw) or ICI (10 µg/paw), µ-, κ- and δ-opioid receptor antagonists respectively, significantly reduced the antinociceptive effect induced by F3 (Figure 6). The antagonists alone did not affect PGE2-induced hyperalgesia (NOR: 70 ± 1.5; ICI: 75 ± 2.1; CTOP: 70 ± 2.5 – data not shown).

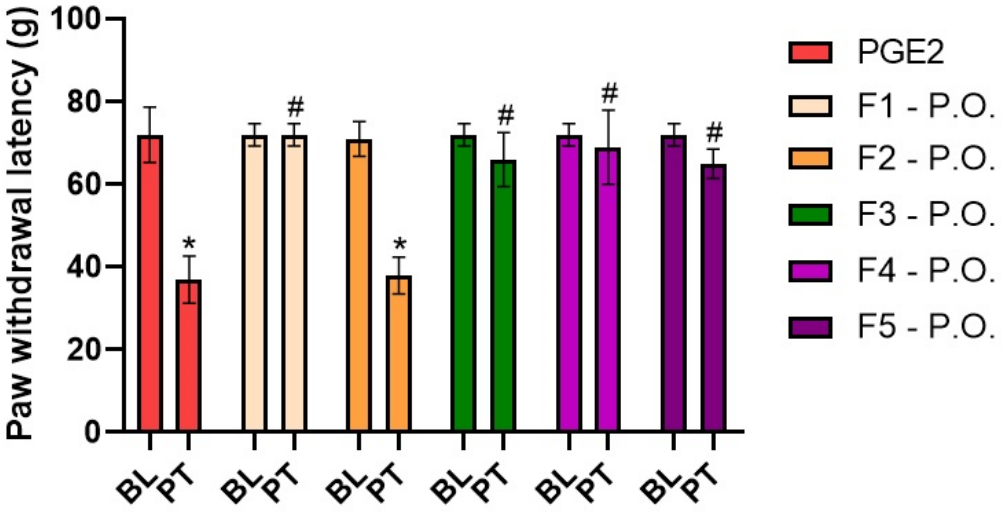

Figure 3: Antinociceptive effects of fractions 1 to 5 on mechanical hyperalgesia induced by intraplantar injection of PGE2. Paw withdrawal latency was assessed before (BL) and 3 hours after intraplantar injection (PT) of PGE2. Fractions (F1 to F5) were administered by intraplantar route immediately before intraplantar injection of PGE2. Data were presented as the mean ± SEM (n=5 rats per group). *p<0.0001 compared to within-subject BL, and #p<0.0001 compared to PGE2 PT. BL: baseline, F1: fraction1, F2: fraction 2, F3: fraction 3, F4: fraction 4, F5: fraction 5, PGE2: prostaglandin E2, P.O.: oral route, and PT: post treatment.

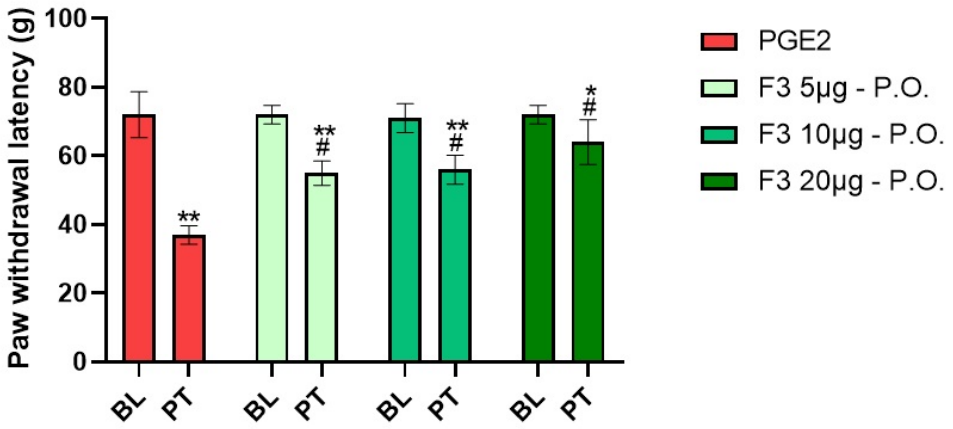

Figure 4: Antinociceptive effect of fraction 3 doses isolated from *Naja kaouthia* venom on mechanical hyperalgesia induced by PGE2. Paw withdrawal latency was assessed before (BL) and 3 hours after intraplantar injection (PT) of PGE2. F3 doses were administered by oral route immediately before intraplantar injection of PGE2. Data were presented as the mean ± SEM (n=5 rats per group). *p<0.05 and **p<0.0001 compared to within-subject BL, and #p<0.0001 compared to PGE2 PT. BL: baseline, F3: fraction 3, PGE2: prostaglandin E2, and PT: post treatment.

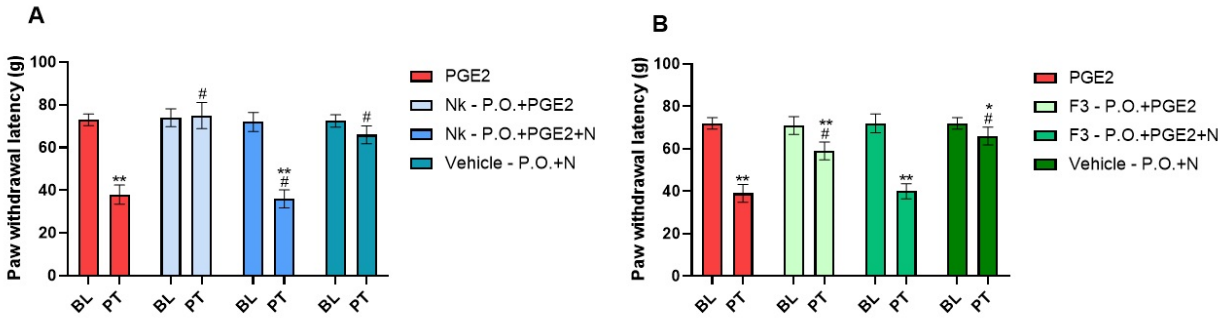

Figure 5: Effect of naloxone on the antinociception induced by *Naja kaouthia* venom and fraction 3. Paw withdrawal latency was assessed before (BL) and 3 hours after intraplantar injection (PT) of PGE2. Nk **(A)** and F3 **(B)** were administered by oral route immediately before intraplantar injection of PGE2. Naloxone was injected into the hind paw 15 minutes before mechanical testing. Data were presented as the mean ± SEM (n=5 rats per group). Graph A: **p<0.0001 compared to within-subject BL, and #p<0.0001 compared to PGE2 PT. Graph B: *p<0.05 and **p<0.0001 compared to within-subject BL, and #p<0.0001 compared to PGE2 PT. BL: baseline, F3: fraction 3, N: naloxone, Nk: *Naja kaouthia* venom, PGE2: prostaglandin E_2, and PT: post treatment.

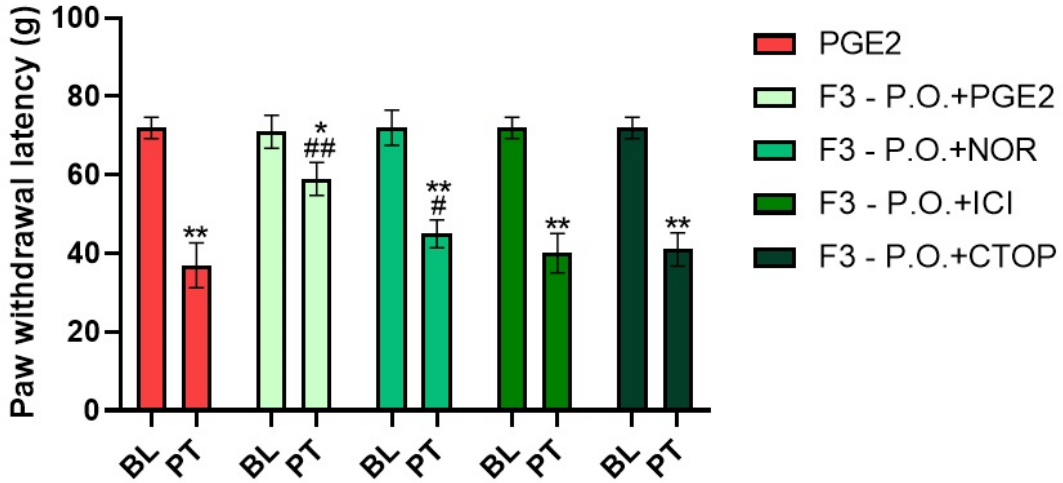

Figure 6: Effects of μ-, κ- and δ-opioid receptor antagonists on the antinociception induced by fraction 3. Paw withdrawal latency was assessed before (BL) and 3 hours after intraplantar injection (PT) of PGE2. F3 was administered by oral route immediately before intraplantar injection of PGE2. The antagonists NOR (κ-opioid receptor antagonist), ICI (δ-opioid receptor antagonist) and CTOP (μ-opioid receptor antagonist) were injected into the hind paw 15 minutes before mechanical testing. Data were presented as the mean ± SEM (n=5 rats per group). *p<0.0005 and **p<0.0001 compared to within-subject BL, and #p<0.05 and ##p<0.0001 compared to PGE2 PT. BL: baseline, CTOP: D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide, F3: fraction 3, ICI: N,N-diallyl-Tyr-Aib-Aib-Phe-Leu, NOR: nor-binaltorphimine, PGE2: prostaglandin E2, and PT: post treatment.

DISCUSSION

In the present study, we first showed that the *Naja kaouthia* venom can decrease pain-related responses in an inflammatory pain model induced by PGE2 in rats when administered by oral or intraplantar routes. Since the oral administration route may be clinically preferred over the various other administration routes due to its advantages, including safety, good patient compliance, ease of ingestion, and pain avoidance, we decided to test the effects of the venom fractions using the oral route only. The use of purified snake venom fractions instead of the whole venom has been widely advised, since this approach enables us to identify the specific proteins present in fractions that may be relevant for the disease being investigated and would be expected to reduce the occurrence of adverse side-effects (Mohamed Abd El-Aziz et al, 2019). We showed that fractions 1, 3, 4 and 5 of the *Naja kaouthia* venom administered by oral route decreased PGE2-induced pain sensitivity, while the fraction 2 did not modify pain-related responses. Even though we did not evaluate differences in proteins or other molecular components of the five fractions, these findings suggest that fractions of the *Naja kaouthia* venom have differential effects on pain sensitivity.

The lowest dose of the fraction 3 appeared to be the most effective dose to reduce PGE2-induced pain sensitivity when administered by the oral route. Thus, we further assessed the effects of naloxone, a non-specific opioid receptor antagonist, on the pain relief induced by the *Naja kaouthia* venom and its fraction 3 only. The antinociceptive effects of the *Naja kaouthia* venom and fraction 3 was abolished by intraplantar injection of naloxone, suggesting the potential role of peripheral opioid receptors. Similarly, intraplantar injection of naloxone blocked the antinociceptive effect of the Crotalus durissus terrificus venom administered by oral and intraplantar routes on an inflammatory pain model induced by PGE2 and neuropathic pain (Gutierrez et al, 2008; Konno et al, 2008; Zambelli et al, 2014). However, the role of specific opioid receptors may differ in the pain relief induced by venoms of *Naja kaouthia* and Crotalus durissus terrificus. Antagonists of μ- and δ-opioid receptors did not alter the antinociceptive effect of the Crotalus durissus terrificus venom on inflammatory pain, whereas the antagonist of κ-opioid receptors blocked the antinociceptive effect (Picolo et al, 2000; Konno et al, 2008). Previously, we demonstrated that crotalphine, a structural analogue to a novel analgesic peptide that was first identified in the crude venom from the South American rattlesnake Crotalus durissus terrificus, induces a potent and long-lasting anti-nociceptive effect that is mediated by the activation of the peripheral k-opioid receptor in PGE2model and the k- and d- opioid receptors, in chronic constriction injury (Zambelli et al, 2014). Since the antinociceptive effect of both the *Naja kaouthia* venom and its fraction 3 was abolished by naloxone, we decided to only investigate if fraction 3-induced antinociception could be mediated by specific opioid receptors. We showed that intraplantar injection of either μ-, κ- or δ-opioid receptor antagonists blocked the pain relief induced by fraction 3. Thus, it appears that all three opioid receptors may be involved in the analgesic effects of fraction 3. In addition, the findings from the intraplantar administration of opioid receptor antagonists may further suggest a peripheral mechanism for the analgesic effect of fraction 3.

The involvement of the central cholinergic system has been postulated as one of the mechanisms underlying the pain relief promoted by the *Naja kaouthia* venom (Chen et al, 2006; Gong et al, 2015). However, the peripheral factors mediating this effect are still unknown. To the best of our knowledge, this is the first study to show the influence of peripheral opioid receptors on the analgesic response induced by orally administered *Naja kaouthia* venom. Microinjection of the α-neurotoxin isolated from *Naja kaouthia* into the periaqueductal gray, a major opioidergic pain modulatory region, did not elicit an analgesic effect on healthy rats (Chen et al, 2006). Thus, the central opioid system does not appear to be involved in the antinociceptive effect of the *Naja kaouthia* venom (Chen et al, 2006). In addition, these findings suggest that the effects of the *Naja kaouthia* venom may vary depending on the state of the subject, i.e., healthy versus painful states, and routes of administration.

This study had some limitations that reduce the impact of the inferences drawn from these findings. First, the sample size was relatively small (n=5 rats per group for all assays). Future studies with larger sample size would be required to show reproducibility of the findings. We fully acknowledge the preliminary aspect of this study. Secondly, future fractionation techniques should include additional two chromatography steps, MALDI-TOF and sequencing so that the effective compound can be identified with 100% accuracy. Thirdly, we did not evaluate sex differences of the test animals (as opposed to sex of the snakes used to produce the venom) in the effects of the *Naja kaouthia* venom and its fractions. Several studies have shown the influence of sex on pain-related responses and peripheral and central processing of pain (Ji et al, 2007; Da Silva et al, 2018; Da Silva et al, 2021). Fourthly, oral administration of the of the *Naja kaouthia* venom had a relatively fast effect on pain-related behaviors (15 min) and the digestive system may have influenced this effect. Oral administration of *Naja, Ophiophagus* and *Crotalus* venoms have been shown to produce antinociceptive and anti-inflammatory activities during relatively short periods (Giorgi et al, 1993; Pu et al, 1995; Zhu et al, 2013). In addition, Australian snake venoms are thermally stable and resistant to proteolysis, which significantly diminishes the effect of the digestive system (Morrison et al, 1983). Thus, larger sample size, evaluation of the role of sex and replication of the current data and analysis are suggested for future studies. Further research is required to provide evidence of the effectiveness and feasibility of the use of the *Naja kaouthia* venom as a source of potential pain therapy.

CONCLUSIONS

This study contributes to the current knowledge about the use of snake venoms in pain management strategies by showing that the *Naja kaouthia* venom and its fractions, particularly fraction 3, may be a potential therapeutic target for pain management and peripheral opioid receptors may mediate the pain relief induced by fraction 3. To the best of our knowledge, this is the first study demonstrating the effects of oral administration of the *Naja kaouthia* venom and its fractions on inflammatory pain in rats, as well as the potential role of peripheral mechanisms. These results significantly highlight an affordable potential target for a non-addictive and non-opioid analgesic treatment that could be used for pain management

in patients. More research is needed to better understand the mechanisms underlying the use of the *Naja kaouthia* venom and its fractions in preclinical models of chronic pain, as well as their clinical implications in different chronic pain conditions.

ACKNOWLEDGMENTS

This work was supported by the São Paulo Research Foundation (grant numbers FAPESP 2017/05218-5; CNPq 405853/2018-1) and Venom Supplies Pty Ltd. Lastly, we would like to pay our gratitude and respects to our colleagues, F. Madaras and Dr. P. Masci for helping conduct this study, who have since passed away.

COMPETING INTERESTS

The authors have no competing interests.

LIST OF ABBREVIATIONS

BL: baseline, CTOP: D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide, F1: fraction1, F2: fraction 2, F3: fraction 3, F4: fraction 4, F5: fraction5, ICI: N,N-diallyl-Tyr-Aib-Aib-Phe-Leu, i.pl.: intraplantar routes, Nk: Naja kaouthia venom, NOR: nor-binaltorphimine, PGE2: prostaglandin E2, P.O.: oral route, PT: post treatment.

ETHICAL STATEMENT

This study received ethical approval from the Institutional Animal Care Committee of the Butantan Institute (CEUAIB, protocol number 248/2005).

AVAILABILITY OF DATA AND MATERIALS

All data will be available upon request.

AUTHORSHIP CONTRIBUTION STATEMENT

All authors were involved with drafting the article or revising it critically for important intellectual content and all authors approved the final version to be published. Drs. Da Silva and Freitas had full access to all of the data and take responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design. Da Silva, Freitas, Giorgi, Mirtschin, Chacur. Acquisition of data. Da Silva, Freitas, Flight, Venning, Lavin, Mirtschin, Chacur, Zambelli, Gutierrez. Analysis and interpretation of data. Da Silva, Freitas, Lavin, Mirtschin, Chacur.

REFERENCES

Munawar A, Ali SA and Betzel C. 2018. Snake Venom Peptides: Tools of Biodiscovery. Toxins, 10, 474.

Mohamed Abd El-Aziz T, Soares AG and Stockand JD. 2019. Snake Venoms in Drug Discovery: Valuable Therapeutic Tools for Life Saving. Toxins, 11, 564.

Gomes A, Bhattacharya S and Gomes A. 2010. Anti-arthritic activity of Indian monocellate cobra (Naja kaouthia) venom on adjuvant induced arthritis. Toxicon, 55, 670-673.

Mohamed Abd El-Aziz T, Soares AG and Stockand JD. 2019. Snake Venoms in Drug Discovery: Valuable Therapeutic Tools for Life Saving. Toxins (Basel), 11.

Metzger M. 2021. Potential Ther otential Therapeutic E apeutic Effects of Snak ects of Snake Venom Components on Pain Management in Rheumatoid Arthritis Patients. Bachelor of Science, Portland Satate University.

Xu JM, Song ST, Feng FY, et al. 2006. Cobrotoxin-containing analgesic compound to treat chronic moderate to severe cancer pain: results from a randomized, double-blind, cross-over study and from an open-label study. Oncol Rep, 16, 1077-1084.

Monaelesser F and TC. 1933. British Medical Journal. 1, 716-717.

Hawgood BJ 1992. Pioneers of anti-venomous serotherapy: Dr Vital Brazil. Toxicon, 30, 573-579.

Gutierrez VP, Konno K, Chacur M, et al. 2008. Crotalphine induces potent antinociception in neuropathic pain by acting at peripheral opioid receptors. Eur J Pharmacol, 594, 84-92.

10. Nogueira-Neto FS, Amorim RL, Brigatte P, et al. 2008. The analgesic effect of crotoxin on neuropathic pain is mediated by central muscarinic receptors and 5-lipoxygenase-derived mediators. Pharmacol Biochem Behav, 91, 252-260.

Gong S, Liang Q, Zhu Q, et al. 2015. Nicotinic acetylcholine receptor α7 subunit is involved in the cobratoxin-induced antinociception in an animal model of neuropathic pain. Toxicon, 93, 31-36.

Liang YX, Zhang ZY and Zhang R. 2015. Antinociceptive Effect of Najanalgesin from Naja Naja Atra in a Neuropathic Pain Model via Inhibition of c-Jun NH2-terminal Kinase. Chin Med J (Engl), 128, 2340-2345.

Kulkeaw K, Chaicumpa W and Tapchaisri P. 2007. Proteome and immunome of the venom of the Thai cobra, Naja kaouthia. Toxicon, 49, 1026-1041.

Wongtongkam N, Wilde H and Ratanabanangkoon K. 2005. A study of Thai cobra (Naja kaouthia) bites in Thailand. Mil Med, 170, 336-341.

Chen ZX, Zhang HL, Gu ZL, et al. 2006. A long-form alpha-neurotoxin from cobra venom produces potent opioid-independent analgesia. Acta Pharmacol Sin 27, 402-408.

Giorgi R, Bernardi MM and Cury Y. 1993. Analgesic effect evoked by low molecular weight substances extracted from Crotalus durissus terrificus venom. Toxicon, 31, 1257-1265.

Picolo G, Giorgi R and Cury Y. 2000. Delta-Opioid receptors and nitric oxide mediate the analgesic effect of Crotalus durissus terrificus snale venom. Eur J Pharmacol, 391, 55-62.

Jose Chirayil C, Abraham J and Thomas S. 2017. Chapter 1 - Instrumental Techniques for the Characterization of Nanoparticles. Thermal and Rheological Measurement Techniques for Nanomaterials Characterization. Elsevier, 1-36.

Picolo G, Cassola AC and Cury Y. 2003. Activation of peripheral ATP-sensitive K+ channels mediates the antinociceptive effect of Crotalus durissus terrificus snake venom. Eur J Pharmacol, 469, 57-64.

Konno K, Picolo G, Gutierrez VP, et al. 2008. Crotalphine, a novel potent analgesic peptide from the venom of the South American rattlesnake Crotalus durissus terrificus. Peptides, 29, 1293-1304.

Picolo G and Cury Y. 2004. Peripheral neuronal nitric oxide synthase activity mediates the antinociceptive effect of Crotalus durissus terrificus snake venom, a delta- and kappa-opioid receptor agonist. Life Sci, 75, 559-573.

Deuis JR, Dvorakova LS and Vetter I. 2017. Methods Used to Evaluate Pain Behaviors in Rodents. Front Mol Neurosci, 10, 284.

Kayser V. 2013. Randall-Selitto Paw Pressure Test. Encyclopedia of Pain, 3357-3360.

Zambelli VO, Fernandes AC, Gutierrez VP, et al. 2014. Peripheral sensitization increases opioid receptor expression and activation by crotalphine in rats. PLoS One, 9, e90576.

Ji Y, Murphy AZ and Traub RJ. 2007. Estrogen modulation of morphine analgesia of visceral pain in female rats is supraspinally and peripherally mediated. J Pain, 8, 494-502.

Da Silva JT, Zhang Y and Seminowicz DA. 2018. Diffuse noxious inhibitory controls and brain networks are modulated in a testosterone-dependent manner in Sprague Dawley rats. Behav Brain Res, 349, 91-97.

Da Silva JT, Tricou C and Ro JY. 2021. Pain modulatory network is influenced by sex and age in a healthy state and during osteoarthritis progression in rats. Aging Cell, e13292.

Pu XC, Wong PTH and Gopalakrishnakone P. 1995. A novel analgesic toxin (hannalgesin) from the venom of king cobra (Ophiophagus hannah). Toxicon, 33, 1425-1431.

Zhu KZ, Liu YL and Qin ZH. 2013. Antinociceptive and anti-inflammatory effects of orally administrated denatured naja naja atra venom on murine rheumatoid arthritis models. Evid Based Complement Alternat Med, 2013, 616241.

Morrison J, Pearn J and Halliday W. 1983. Immunological stability of an elapid venom, tropidechis carinatus, and its relevance to the clinical detection of snake venom. Australian Journal of Experimental Biology and Medical Science, 61, 489-495.