Analgesic effect of morphine and tramadol in standard toxicity assays in mice injected with venom of the snake *Bothrops asper*

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Short title: Analgesia in envenomed mice

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**ABSTRACT**

Routine laboratory animal tests necessary to assess the toxicity of snake venoms and the preclinical neutralizing ability of antivenoms and other inhibitory substances induce significant pain and distress. This has prompted initiatives to introduce the routine use of analgesia. In this study, the analgesic effect of morphine and tramadol was assessed in tests assessing the lethal, hemorrhagic, myotoxic and edema-forming activities of the venom of the viperid snake *Bothrops asper*. The Mouse Grimace Scale (MGS) and mouse-exploration activity were used to assess pain and its inhibition by the analgesics. Results demonstrate that tests assessing lethality and myotoxicity induce higher levels of pain than assays quantifying hemorrhagic and edema-forming activities. Our observations also indicate that pretreatment of mice with both analgesics, at the doses used, were similarly effective in reducing the MGS magnitude and increase mouse-exploration activity after the administration of *B. asper* venom. Moreover, the analgesic effect of both drugs was more evident in the myotoxic and lethality assays. Combined with previous observations showing that these analgesics do not alter the extent of toxic effects induced by *B. asper* venom, our results strongly indicate that the use of analgesia (using either morphine or tramadol) should be considered in the routine assessment of venom toxicity and antivenom efficacy.

**KEY WORDS:** Snake venom; *Bothrops asper*; tramadol; morphine; analgesia; mouse grimace scale.

**INTRODUCTION**

Toxicity assays in laboratory animals, mostly mice and rats, is widely used to characterizse the toxicological profile of venoms and toxins of various origins, and is a regulatory requirement to assess the venom-neutralizing efficacy of antivenoms and other inhibitory substances. When injected by various routes, many venoms and toxins induce pain in these animals, particularly venoms that inflict local tissue damage, such as those of most viperid and some elapid snake species (Gutiérrez et al., 2017). For example, whole venom and venom myotoxic phosholipases A2 (PLA2), PLA2 homologues and haemorrhagic metalloproteinases (SVMPs) of *Bothrops asper* cause prominent local pathological effects, including both hyperalgesia and allodynia in rodents (Chacur et al., 2004a, 2004b, 2003, 2001; Fernandes et al., 2007; Teixeira et al., 2003; (Gutiérrez et al., 2009)).

There is widespread concern with the pain and suffering experienced these necessary tests exert upon laboratory animals. In the case of snake venoms, the WHO standard (ref to latest guidelines) for assessing toxicity and antivenom efficacy is the mouse lethality assay, in which venoms (LD50), or venom-antivenom mixtures (ED50), are administered by intravenous (i.v.) or intraperitoneal (i.p.) routes, and lethality is assessed after a predefined time interval (Gutiérrez et al., 2017; WHO, 2010). Regarding the preclinical evaluation of antivenom efficacy, in addition to the ED50 assay, the WHO Guidelines for the Production, Control and Regulation of Antivenom Immunoglobulins also recommend tests to measure the neutralization of other toxic pathologies, such as hemorrhage, myotoxicity, dermonecrosis and defibrinogenation (WHO, 2010), all of which cause pain.

The toxinological community has researched *in vitro* alternatives of *in vivo* testing of venom toxicity and antivenom efficacy (see Sells, 2003; Gutiérrez et al., 2017), within the domain of Reduce, Replace and Refine (3Rs) in experimental biomedicine (Robinson, 2005; Russel and Burch, 1959). However, the complex multi-organ and multi-physiological system pathology of snakebite envenoming is extraordinarily difficult to validly using *in vitro* assays. Moreover, the preclinical venom LD50 and antivenom ED50 assays remain a global regulatory requirement. Hence, the use of laboratory animals will continue in the field of Toxinology.

The use of analgesia is an obvious means to reduce the pain and distress of animals injected with venoms or toxins (Holmes et al., 2010; Madden et al., 2012), and some advances have been made in the field of snake venoms. Harris et al., (2013) reported the use of buprenorphine, a µ-selective opioid, in their studies on myotoxic activity of snake venoms and toxins. More recently, Gutiérrez and Herrera (2014) described that morphine and tramadol, at doses previously shown to have analgesic effects in mice, did not affect the magnitude of various hemorrhagic, myotoxic, edema-forming and defibrinogenating effects upon mice when compared to animals not receiving these analgesics. Furthermore, Chacón et al. (2015) showed that the use of tramadol analgesia did not affect the result of antivenom ED50 assays in mice receiving *B. asper* venom.

Measuring pain in animals is clearly difficult but is necessary to demonstrate analgesic interventions. Thus, while the studies above have importantly shown that analgesic drugs did not significantly affect measurable outputs of preclinical testing, they lack quantitative evidence that analgesics reduced pain in the animals. To this end, we used the Mouse Grimace Scale (Langford et al, 2010) and mouse-exploration activity as tools to measure the analgesic benefits of tramadol and morphine in mice injected with doses of *B. asper* venom routinely used in preclinical tests. Our results demonstrate that these analgesics confer significant analgesic benefit to experimental mice and provide impetus for wider introduction of analgesia in the routine assessment of venom toxicity and antivenom efficacy.

**MATERIALS AND METHODS**

**Venom**

*Bothrops asper* venom was a pool obtained from more than 40 adult specimens collected in the Pacific region of Costa Rica. It was lyophilized and stored at -40°C until used.

**Mice**

CD-1 mice (*Mus musculus*) with a body weight between 18 and 20 g were used in this study. The experimental protocols were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUA) of the University of Costa Rica (CICUA 078-17) and meet the International Guiding Principles for Biomedical Research Involving Animals (CIOMS). Mice were maintained under standard conditions of temperature (22±2˚C), light/dark cycles of 12 h, and received food and water *ad libitum*.

**Study design**

After basal measurement of analgesia and mouse activity, as described below, groups of five mice were pre-treated with either 0.12 M NaCl, 0.04 M phosphates, pH 7.2 (PBS), morphine (10 mg/kg) or tramadol (50 mg/kg) by the subcutaneous route. The doses of the analgesics were selected according to previous studies in which these substances were used (Díaz-Reval et al., 2010; Gades et al., 2000; Gutiérrez and Herrera, 2014; Kissel et al., 1961; Raffa et al., 1992; Umans and Inturrisi, 1981). Fifteen minutes after the application of analgesics or PBS, animals were injected with *B. asper* venom according to the various protocols used for the assessment of toxic activities.

To determine the analgesic effect in the hemorrhagic activity assay, mice were injected with 20 μg of venom, dissolved in 0.1 mL of PBS, by intradermal (i.d) route in the ventral abdominal region (Gutiérrez et al., 1985; Gutiérrez and Herrera, 2014). In the case of the edema-forming activity assay, mice were injected with 5 μg of venom, dissolved in 0.05 mL of PBS, in the right foot pad by subcutaneous (s.c.) route (Chaves et al., 1995; Gutiérrez and Herrera, 2014). For the myotoxic activity assay, mice were injected intramuscularly (i.m.) with 50 μg of venom dissolved in 0.1 mL of PBS, in the right gastrocnemius (Gutiérrez et al., 1980; Gutiérrez and Herrera, 2014). Finally, for the lethality assay, mice received intraperitoneal (i.p.) injections of a sub-lethal dose of 44 μg of venom, dissolved in 0.2 ml of PBS, which is equivalent to 0.75 times the LD50 (Chacón et al., 2015; Rucavado et al., 2004).

After venom administration, the analgesic effect and mouse activity was determined at different time intervals, as described below. Mice were observed for 2 h in the case of the hemorrhagic activity test, 3 h for the myotoxic activity test, and 6 h for the edema-forming activity and lethal activity tests, according to the corresponding duration of each assay.

**Mouse Grimace Scale (MGS)**

Mice were placed individually in transparent acrylic boxes (9 × 5 × 5 cm high), with three opaque sides walls, and a digital video camera was positioned in front of the transparent wall. Animals were acclimated to the box for 10 minutes and then filmed for 30 seconds before the administration of any treatment (i.e. basal measurement), and this repeated at different time points after venom administration to mice previously treated with either PBS or one of the analgesics.

A random picture from each video was taken in JPEG format and five facial features (i.e. orbit tightening, cheek bulge, nose bulge, ear position and whisker position) were scored with a value of 0 (not present), 1 (moderately visible) or 2 (severe), according to the “Mouse Grimace scale” (MGS) proposed by Langford et al. (2010). Images were randomized for analysis, and the coder was blinded to the treatment.

The final MGS score was the average of the five feature units, and the MGS difference score was obtained for each mouse at each time point by subtracting the basal MGS score from the MGS score after venom administration.

**Mouse exploration activity**

Groups of five mice were placed in an open field (41 x 41cm) with a gridded surface (14 x 13.5 cm) and plastic transparent walls. Animals were filmed for 30 s before the administration of any treatment (i.e. basal measurement), and then at different times points after venom injection in mice that had received either PBS or one of the analgesics, until the end of the test. The videos were analyzed to determine the number of head movements performed during 30 s for each mouse, as a measure of activity score. Also, the number of frames crossings performed during 30 s was determined for each mouse, as a measure of locomotor activity (Carola et al., 2002).

**Statistical analyses**

Results were expressed as the mean ± standard error of the mean (SEM). Normality and homoscedasticity of all data sets were confirmed by using the Shapiro-Wilk and Levene test, respectively. A one-way analysis of variance (ANOVA) with repeated measures, followed by Dunnet as *post hoc* test, was performed in the control group to compare baseline with data after venom administration to determinate if venom administration causes changes in each parameter throughout the model. A two-way repeated measures ANOVA, with the time points as the within-subjects factor and the treatment group as the between-subjects factor, and Bonferroni as *post hoc* test, were performed to compare the treatment groups with the control group at each time point after venom administration to determine the analgesic effect and the effect on the mouse activity throughout the model. A p-value < 0.05 was considered significant. All statistical analyses were performed by using the IBM SPSS Statistics 22.0 and GraphPad Prism 6 software.

**RESULTS**

**Mouse Grimace Scale**

To determine whether *B. asper* venom administration increases the MGS score in the various assays, we compared the difference between the MGS score before (basal) and after venom administration (i.e. MGS difference score) with zero in the control group (i.e. non-analgesic treatment) at each time point by one-way repeated measures ANOVA, followed by Dunnet test.

For the hemorrhagic assay (Fig. 1A), the MGS difference score was significantly different from zero in the first 30 min after venom injection (p < 0.01). In the edema-forming activity assay (Fig. 1B), the increase of the MGS difference score was also significantly different from zero value at 6 h after venom administration (p<0.05). For the rest of the time points, the MGS difference score for the control group was not significantly different to zero in these assays; however, there was a trend to increase the MGS difference score as compared to the baseline. In contrast, for myotoxicity (Fig. 1C) and lethality (Fig. 1D) assays, the MGS difference scores in the venom control groups were significantly different to zero over the duration of the tests (p<0.001), i.e. 3 h and 6 h, respectively. Fig 1E shows representative images for the lethality assay, 30 min after i.p. administration of a sublethal dose of *B. asper* venom. The venom control mouse shows a facial grimace of pain as compared to baseline with the following features: narrowing of the orbital area, contracted cheek muscle, the presence of a bulge on the top of the nose, and the ears and whiskers are pulled back.

Next, to determine whether the pain induced by *B. asper* venom administration in the different assays was reduced by morphine and tramadol treatment (i.e. decrease the MGS difference score), we compared the MGS difference score between treatment groups at each time point by two-way repeated measures ANOVA, followed by Bonferroni test. Time, treatment and time\*treatment interaction had significant effects on the MGS differences score for the myotoxic and lethality assays, but not for the hemorrhagic activity and edema-forming activity assays. Morphine and tramadol significantly decreased the MGS difference score at 30 and 90 min after venom administration in the myotoxicity and lethality assays (see Fig 1C y 1D for p values). Additionally, tramadol significantly decreased the MGS difference score 3 h after venom administration in the myotoxicity assay (p < 0.01). For the rest of the time points, the MGS difference score for morphine and tramadol was not significantly different as compared to venom control; however, there is a trend to decrease the MGS difference score in animals receiving analgesics. Moreover, no significant difference in the effect on the MGS score after *B. asper* venom administration was found between morphine and tramadol at any individual time points throughout the study. Fig. 1E shows representative images for the analgesic effect of morphine and tramadol as compared to venom control for the lethality assay, 30 minutes after *B. asper* venom administration. Envenomed mice previously treated with either morphine or tramadol show a facial grimace with similar features as compared to baseline. For the hemorrhagic (Fig. 1A) and edema-forming (Fig. 1B) assays, there is a trend for morphine and tramadol to decrease the MGS difference score as compared to control, but it was not statistically significant.

**Activity Score**

The basal activity score (i.e. prior to analgesic and venom administration) was not significantly different between groups of mice used in the different assays, so the basal mean for 60 mice was calculated as a reference value, which is indicated with a horizontal dotted line in Fig. 2. This value was 19 ± 4 head movements during 30 s. Nevertheless, the statistical analysis was carried out with the basal values for each experiment.

*B. asper* venom administration significantly decreased the head movements in control mice over the duration of the tests for edema-forming, myotoxic and lethal activities (see Fig 2B, 2C y 2D for p values). However, in the hemorrhagic activity assay, the head movements significantly decreased 2 h after *B. asper* venom administration (p < 0.05), but not at 30 and 90 min (Fig 2A).

Treatment with both analgesics reversed the decrease in head movements induced by administration of *B. asper* venom in some time points in the different assays, especially when testing the edema-forming, myotoxic and lethal activities (Fig. 2B, 2C, 3C). For example, morphine and tramadol significantly increased the head movements as compared to venom control over the duration of the lethality and myotoxic activity assays (see Fig 2C y 2D for p values). In the edema-forming activity assay, the effect of morphine and tramadol was observed during the first 2 h (see Fig 2B for p values). In the hemorrhagic activity assay, morphine, but not tramadol, significantly increased the head movement as compared to control at 2 h (p < 0.01) (Fig. 2A).

**Locomotor Activity**

As described for the activity score, there were no significant differences for the basal locomotor activity between groups of mice from different assays, so the basal mean for 60 mice was calculated as a reference value. This value was 18 ± 6 crossings in a grid table during 30 s, and it is indicated with a horizontal dotted line in Fig. 3. However, the statistical analysis was carried out with the basal values for each experiment.

In the hemorrhagic and edema-forming activity assays, the administration of *B. asper* venom significantly decreased the crossings in the control groups at some time points (see Fig. 3A and 3B for p values). However, there was a trend to decrease the crossings in the control groups over the duration of these tests. On the other hand, *B. asper* venom administration significantly decreased the crossings in control mice over the duration of the tests for myotoxic activity and lethality (see Fig 3C y 3D for p values).

The treatment with both analgesics reversed the decrease in crossings induced by administration of *B. asper* venom at some time points in the different assays. Tramadol significantly increased the crossings as compared to venom control at 30 min (p < 0.01) in the hemorrhagic activity assay, and at 60 min in the edema-forming activity (p < 0.01), myotoxic activity (p < 0.01) and lethality assay (p < 0.001). On the other hand, morphine significantly increased the crossings as compared to venom control during the first 90 min, 2 h y 4 h in the hemorrhagic activity, lethality and edema-forming activity assays, respectively (see Fig 3A, 3B y 3D for p values).

**DISCUSSION**

This study demonstrates that morphine and tramadol, at the doses used, are effective analgesics in standard preclinical assays used to assess the lethal, hemorrhagic, myotoxic and edema-forming activities of the venom of *B. asper*. It has been previously shown that the same analgesics, at the same doses used here, do not affect the estimation of these toxic effects in this venom (Gutiérrez and Herrera, 2014). These findings provide strong evidence supporting the use of analgesia in the assessment of snake venoms activities and their neutralization by antivenoms and a variety of inhibitors.

Pain in mice usually results in decreased exploration and locomotor activity, as well as in changes in some behavioral patterns, which have been used to assess pain in this species (Miller et al., 2011; Wright-Williams et al., 2007). However, these features are not exclusive of pain behavior since they also reflect distress and anxiety in mice. The Mouse Grimace Scale developed and validated by Langford et al. (2010) provided a specific tool to assess pain induced by different nociceptive procedures (Leach et al., 2012; Miller et al., 2016; Mittal et al., 2016) and to evaluate the efficacy of analgesic drugs (Matsumiya et al., 2012; Miller et al., 2015; Miller and Leach, 2015; Roughan et al., 2016). These studies demonstrated that the MGS is an accurate and reproducible measure of pain, and requires only a short period of training for the evaluator. Our results show an increase in the MGS and a decrease in the exploration activity in the control mice after the administration of *B. asper* venom, which demonstrates the presence of pain in the different toxicity assays over time. Moreover, our findings indicate that, in the experimental settings used, myotoxic and lethality activities assays are more painful than hemorrhagic and edema-forming activities assays, since the MGS difference score was higher in the venom control mice and significantly different to baseline for most of the times. For the hemorrhagic activity test the increase in the MGS difference score was significantly different to baseline only in the first 30 min of the assay, and the exploration activity was less affected during this assay, which indicates that it is less painful for the animal.

Our results evidence that pretreating mice with morphine and tramadol prevented the increase in the MGS and the decrease in the exploration activity of mice injected with *B. asper* venom, results consistent with the mechanism of action of these analgesics. Morphine and tramadol are potent and weak agonists of opioid receptors (µ, δ and κ), respectively. They act directly on the central nervous system, at spinal and supraspinal regions, to modulate and decrease the transmission of pain (Pasternak, 1993; Yaksh, 1997). Tramadol has no effect on respiratory or cardiovascular function, and has a low potential to generate tolerance (Raffa et al., 1992; Scott and Perry, 2000), which is an advantage over morphine in the context of snake venom studies. Moreover, our results indicate that morphine, but not tramadol, may increase the locomotor activity in mice, in agreement with earlier reports (Carroll and Sharp, 1972; Kuribara, 1997; Murphy et al., 2001; Parker, 1974). Previous studies have demonstrated that morphine and other opioids do not induce changes in facial expression in pain-free animals (Leach et al., 2012; Matsumiya et al., 2012), implying that the effects observed in the MGS are due to the analgesic effect.

Although morphine is theoretically a more potent analgesic than tramadol, our results show that both drugs, at the doses used, are similarly effective in the relief of pain induced by *B. asper* venom in the different toxicity assays. Moreover, the analgesic effect of both drugs was more evident in the myotoxic and lethality tests, which were the most painful assays, and the effect was sustained during the whole observation period for most of the assays. This agrees with the duration of the action of these analgesics in mice which have been estimated to be between 2 to 3 hours for morphine (Gades et al., 2000; Umans and Inturrisi, 1981) and up to 6 hours for tramadol (Evangelista Vaz et al., 2017). Therefore, these analgesics are useful for short-term toxicological tests in which results are assessed within few hours after venom or toxin administration. The duration of the hemorrhagic, myotoxic and edema-forming activities assays in this study corresponded to those used in the routine toxicity tests for the assessment of venom toxicity and antivenom efficacy. However, in the lethality test the observations were performed during the first 6 hours, while the test lasts 24 or 48 hours. Therefore, more extended observation periods are necessary to decide whether additional injections of analgesics are required in the lethality assay. Buprenorphine has a more prolonged duration of action, estimated to be between 3 to 5 h in mice (Gades et al., 2000). Thus, this analgesic could be a better option for the lethality test to reduce the number of injections during the assay; however, potential interferences of this drug with the lethality test should be studied.

In conclusion, our results demonstrate that both morphine and tramadol are effective mouse analgesics in routine preclinical testing, hereby shown for the case of *B. asper* venom. Since the mechanisms of toxicity and the causative toxins are generally similar in all viperid snake venoms, it is suggested that our findings may be applicable to the study of other venoms, an issue that demands further investigation. Taken together, these results and those of a previous study showing that these analgesics do not alter the results of toxicity assays in the case of *B. asper* venom (Gutiérrez and Herrera, 2014) support the routine introduction of analgesia in the study of venoms toxicity and in the evaluation of the preclinical efficacy of antivenoms.

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**Figure legends**

**Fig. 1. Effect of morphine and tramadolon the mouse grimace scale (MGS) score after *B. asper* venom administration.** Groups of mice (n = 5) were injected by subcutaneous route with either morphine (10 mg/kg), tramadol (50 mg/kg) or PBS (control). After fifteen minutes, mice were injected with *B. asper* venom according to the **(A)** hemorrhagic activity assay (20 µg intradermal), **(B)** myotoxic activity assay (50 µg intramuscular), **(C)** edema-forming activity assay (5 µg intraplantar) and **(D)** lethality assay (45 µg intraperitoneal), as described in methods. The MGS score was determined prior any analgesic and venom administration (basal), and after venom administration at different times. Results are expressed as the mean ± SEM of the difference between the MGS score after venom administration and the basal MGS score for each mouse (i.e. MGS difference score). tp < 0.05, ttp < 0.01, tttp < 0.001 compared to zero by one-way repeated measures ANOVA, followed by Dunnet test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 compared to control group at each time point by a two-way repeated measures ANOVA, followed by Bonferroni test. **(E)** Representative images for the lethality assay prior any analgesic and venom administration (basal), and 30 minutes after *B. asper* venom administration in mice pretreated with either analgesic (i.e. morphine or tramadol) or PBS (control). For representative images of each feature in the MGS, see reference Langford et al. (2010).

**Fig. 2. Effect of morphine and tramadolon the activity score after *B. asper* venom administration.** Groups of mice (n = 5) were injected by subcutaneous route with either morphine (10 mg/kg), tramadol (50 mg/kg) or PBS (control). After fifteen minutes, mice were injected with *B. asper* venom according to the **(A)** hemorrhagic activity assay (20 µg intradermal), **(B)** myotoxic activity assay (50 µg intramuscular), **(C)** edema-forming activity assay (5 µg intraplantar) and **(D)** lethality assay (45 µg intraperitoneal), as described in methods. The activity score (i.e. head movements during 30 s) was determined prior any analgesic and venom administration (basal), and after venom administration at different times. Results are expressed as the mean ± SEM. The horizontal dotted line represents the mean basal value of 60 mice. tp < 0.05, ttp < 0.01, tttp < 0.001 compared to basal data by one-way repeated measures ANOVA, followed by Dunnet test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 compared to control group at each time point by a two-way repeated measures ANOVA, followed by Bonferroni test.

**Fig. 3. Effect of morphine and tramadolon the locomotor activity after *B. asper* venom administration.** Groups of mice (n = 5) were injected by subcutaneous route with either morphine (10 mg/kg), tramadol (50 mg/kg) or PBS (control). After fifteen minutes, mice were injected with *B. asper* venom according to the **(A)** hemorrhagic activity assay (20 µg intradermal), **(B)** myotoxic activity assay (50 µg intramuscular), **(C)** edema-forming activity assay (5 µg intraplantar) and **(D)** lethality assay (45 µg intraperitoneal), as described in methods. The locomotor activity (i.e. crossings in a grid table during 30 s) was determined prior any analgesic and venom administration (basal), and after venom administration at different times. Results are expressed as the mean ± SEM. The horizontal dotted line represents the mean basal value of 60 mice. tp < 0.05, ttp < 0.01, tttp < 0.001 compared to basal data by one-way repeated measures ANOVA, followed by Dunnet test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 compared to control group at each time point by a two-way repeated measures ANOVA, followed by Bonferroni test.