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Letter to the Editor

The analgesics morphine and tramadol do not alter the acute toxicity induced by *Bothrops asper* snake venom in mice

Experimental Toxinology, i.e. the study of the composition and mechanisms of action of naturally-derived venoms or toxins at the experimental level, is a scientific field that involves, among diverse methodological tools, the analysis of the actions of venoms or toxins in animals, most frequently rodents or lagomorphs, but also in larger species. Administration of venoms or toxins generally causes pain. A typical example is the use of viperid snake venoms, or purified tissue-damaging toxins from these venoms. For instance, the venom of the pit viper *Bothrops asper*, as well as myotoxic PLA_2s and PLA_2 homologs, and hemorrhagic SVMPs isolated from this venom, provoke pain, i.e. hyperalgesia and allodynia, in rodents (Chacur et al., 2001, 2003, 2004a, 2004b; Teixeira et al., 2003; Fernandes et al., 2007). This is also the case with many types of animal venoms and toxins and microbial toxins which induce soft tissue necrosis and inflammation, and systemic alterations associated with pain.

There is a widespread and growing concern with the suffering, specifically the generation of pain, that experimental animals undergo in tests designed to evaluate the toxicological profile and mechanism of action of venoms and toxins, and the ability of therapeutic agents, such as antivenoms, to neutralize these effects. In particular, the preclinical assessment of the neutralizing ability of antivenoms involves the injection of venoms, or of mixtures of venoms and antivenoms, to experimental animals, mostly mice, with the consequent generation of distress and pain. In addition to the gold standard for evaluating antivenom efficacy, i.e. the assay for neutralization of lethality in mice, the WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins (WHO, 2010) include various additional assays for the analysis of the preclinical efficacy of antivenoms, in order to have a more integrated view of the neutralizing profile of these immunotherapeutics. These are the neutralization of hemorrhagic, myotoxic, edema-forming, dermonecrotic and defibrinogenating activities, which characterize envenomings by viperid venoms and, in some cases, by elapid snake venoms as well (Theakston and Reid, 1983; WHO, 2010; Gutiérrez et al., 2013).

The reduction of pain in these assays, as part of the general ethical framework of replacement, reduction and refinement of the laboratory methods performed in animals (Russell and Burch, 1959; Robinson, 2005) should receive more attention from the toxinological research community. The refinement, defined as any approach that reduces or eliminates the potential pain or distress in animals, and which enhances animal wellbeing, is of particular importance in toxicity testing (Holmes et al., 2010; Madden et al., 2012). One of the reasons why toxinologists have been slow in the introduction of precautionary analgesia in experimental research has to do with the concern that this intervention might affect the results of the assays. In this context, proper validation of the test method is necessary to demonstrate that the alternative proposed is scientifically valid and generates the same results (Schechtman, 2002). Recently, Harris et al. (2013) highlighted the need to perform research on the use of analgesics in experimental Toxinology. They described the experience gained in their laboratory for many years with the use of the μ-selective opioid buprenorphine. In this communication we present results on the use of the analgesics morphine and tramadol in the study of hemorrhagic, myotoxic, edema-forming and defibrinogenating activities of *B. asper* venom in mice, and whether the use of these analgesics affects the outcome of the toxinological tests performed.

In all cases, groups of CD-1 mice (18–20 g) were pretreated with either distilled water or analgesics (either morphine sulfate (Laboratorio Sanderson S.A., Chile, 5 and 10 mg/kg) or tramadol chlorhydrate (Laboratorio Sanderson S.A., Chile, 50 mg/kg) by the subcutaneous route). The doses of analgesics used were selected on the basis of previous studies estimating the analgesic effect of these drugs in mice (Kissel et al., 1961; Umans and Inturrisi, 1981; Raffa et al., 1992; Gades et al., 2000; Díaz-Reval et al., 2010). Fifteen min after administration of either analgesic or vehicle (water), animals were injected with *B. asper* venom

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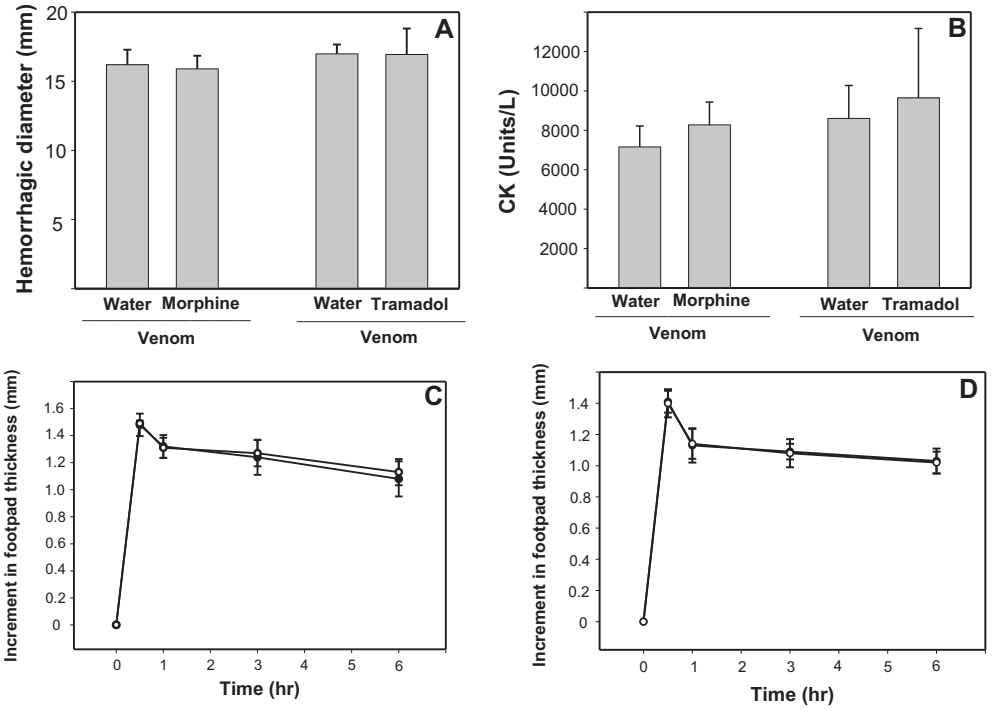

Fig. 1. Hemorrhagic (A), myotoxic (B), and edema-forming (C, D) activities of *Bothrops asper* venom in mice. Groups of mice were injected subcutaneously with either morphine (5 mg/kg), tramadol (50 mg/kg) or water, in a total volume of 100 μL. Fifteen min afterward, animals were injected with *B. asper* venom for the assessment of hemorrhagic, myotoxic and edema-forming activities, as described in the text. In the case of edema-forming activity, (C) shows the time-course of the effect in mice pretreated with either water or morphine, whereas (D) shows the results in mice pretreated with either water or tramadol. Results are presented as mean ± S.D. ($n = 5$). No significant differences ($p > 0.05$) were observed in any of the effects between mice pretreated with water and those pretreated with the analgesics.

for the assessment of each effect. For hemorrhagic activity, 20 μg of venom, dissolved in 100 μL of 0.14 M NaCl, 0.04 M phosphate, pH 7.2 (PBS), were injected intradermally in the ventral abdominal region of mice ($n = 5$). Two hr after injection, animals were sacrificed by CO_2 inhalation, their skins were removed and the diameter of the hemorrhagic halo in the inner side of the skin was measured (Gutiérrez et al., 1985). For myotoxic activity, similar groups of mice ($n = 5$) were injected intramuscularly, in the right gastrocnemius, with 50 μg venom dissolved in 50 μL PBS. After 3 h, mice were bled from the tail and the creatine kinase (CK) activity of plasma was determined using a commercial kit (CK LIQUI-UV, Stanbio Lab., Texas, USA) (Gutiérrez et al., 1980). Afterward, animals were sacrificed by CO_2 inhalation, the injected gastrocnemius muscle was dissected out, and tissue samples were immersed in 10% formaldehyde fixative solution. After routine processing, samples were embedded in paraffin, and sections were stained with hematoxylin and eosin for microscopic examination.

For edema-forming activity, mice ($n = 5$) were injected subcutaneously in the right foot pad with 5 μg venom dissolved in 50 μL PBS. The thickness of the footpad was measured with a low-pressure spring caliper (Lomonte et al., 1993) before injection and at various time intervals after injection. Defibrinogenating activity was determined using the method of Theakston and Reid (1983), as modified by Gené et al. (1989). Briefly, groups of mice ($n = 3$) were injected i.v. with various doses of venom, dissolved in 100 μL PBS. One hr after injection, a sample of blood was collected by cardiac puncture under anesthesia with ketamine and xylazine. Two hundred μL of blood were placed in dry glass tubes and allowed to stand at room temperature for 20 min. The Minimum Defibrinogenating Dose (MDD) corresponds to the venom dose at which the blood from the three animals remained unclottable (Gené et al., 1989). In all cases, control mice were injected with the same volume of PBS without venom. Experiments were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUA) of the University of Costa Rica.

As shown in Fig. 1, there were not significant differences in the magnitude of hemorrhagic, myotoxic and edema-forming effects of *B. asper* venom in mice pretreated with either distilled water, or morphine or tramadol at the doses used. In agreement, histological assessment of muscle tissue revealed a similar extent of hemorrhage and myonecrosis in mice pretreated with water or analgesics and then injected with venom (not shown). Likewise, no difference was observed in the estimation of the defibrinogenating effect, since the MDD corresponded to 5 μg in animals pretreated with either water or the analgesics. Mice pretreated with the analgesics showed notoriously less distress and discomfort after envenoming than those