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Research Article

Effects of *Vochysia haenkeana* extract on the neuromuscular blockade induced by *Bothrops jararaca* venom on chick biventer cervicis preparation *in vitro*

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SUMMARY

Vochysia haenkeana extract (*Vh*-E) was assessed against the neuromuscular blockade induced by *Bothrops jararaca* venom on chick biventer cervicis (BC) preparation. Pre- and post-venom incubation treatments (Pre-*vit* and Post-*vit*) were analysed here. Contractures ACh (110 μM) and KCl (20 mM) were evoked before and after addition of venom without stimulation. *Vh*-E (600 μg/mL) under Pre-*vit* was more efficient to neutralize the neuromuscular blockade by venom (40 μg/mL) [72.5±4.6% (venom) *vs*. 45.2±14% (*Vh*-E) of blockade, $p<0.05$, n=4]. *Vh*-E (600 μg/mL) did not cause significant changes under Post-*vit* [72.5±4.6% (venom) *vs*. 63.4±8.2% (*Vh*-E) of blockade, n=4]. The Pre-*vit* inhibited the blockade of the contracture to ACh (106±17% of response; n=4) while the Post-*vit* was able to attenuate the effect of the venom on this contracture (55±5% of response; n=4); related to those contractures to KCl both of treatments with *Vh*-E attenuated the blocker effect of the venom (62.5±7.7% and 55±5% of response for Pre-*vit* and Post-*vit*, respectively; n=4). In conclusion, *Vh*-E neutralizes partially the neuromuscular blockade in Pre-*vit*, an effect that can be related to preserved function of "extrinsic" post-synaptic receptors, by measured contractures in response to ACh. The myotoxicity of the venom was significantly reduced by *Vh*-E in both, Pre-*vit* and Post-*vit*, by measured contractures in response to KCl.

INTRODUCTION

Accidents by snake bites are considered a severe problem of public health in many regions of the world mainly in sub- and tropical countries. Although the serum therapy is an efficient and recommended treatment, the investigation of alternative methods to delay the effects of the envenomation, especially during the first hours, has proved to be relevant [1]. The effects of medicinal plants against snake venoms have been studied as an alternative to support the serum therapy [2-4]. It has been shown that the bioactive compounds from these plants may neutralize the neuromuscular blockade caused by *Bothrops* venoms *in vitro* [5]. In Brazil, especially in some areas of difficult access in the northern of the country, *V. haenkeana* has been popularly used to treat snake bites but without any scientific evidences [6]. In this work, we have investigated the ability of the hydroalcoholic extract from stem barks of *V. haenkeana* to neutralize the neuromuscular effects induced by *Bothrops jararaca* venom on avian neuromuscular preparation *in vitro*.

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MATERIAL AND METHODS

Animals

The animals were housed (5 animals per cage) at 23-25°C on a 12 h light/dark cycle and they had water *ad libitum* and access to appropriate food (Prefran®, Sales Oliveira, SP, Brazil). This project was approved by the institutional Committee for Ethics in Research of University of Sorocaba (UNISO, protocol no. 53/2015) and the experiments were carried out as the established guidelines of Brazilian Society of Laboratory Animal Science (SBCAL).

Extract of *V. haenkeana*

The hydroalcoholic extract from stem barks of *V. haenkeana* (*Vh*-E) was kindly donated by Prof. Dr. Márcio Galdino dos Santos from Tocantins Federal University (UFT, Porto Nacional, TO, Brazil). The methodology used to obtain *Vh*-E is essentially described elsewhere [7]. A voucher specimen 10.074 was identified by Solange de Fátima Lolis from the Biological Sciences Department, of the Tocantins Federal University (UFT, Porto Nacional, TO, Brazil) and deposited in the Herbarium of this university in accordance with the *International Code of Botanical Nomenclature* (ICBN). In all experiments, the *Vh*-E was solubilized in 30 µL of 70% ethanol (Cinética Soluções Químicas, Londrina, PR), as described elsewhere [8].

Bothrops jararaca venom

B. jararaca venom was donated and certified by Prof. Dr. Stephen Hyslop from the Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas (UNICAMP).

Chick biventer cervicis (BC) preparation

Chicks were killed by halothane inhalation and the biventer cervicis muscles were removed [9] and mounted under a tension of 1 g in a 5 mL organ bath (Panlab® four chamber organ bath) containing aerated (95% O_2/5% CO_2) Krebs solution (composition, in mM: NaCl 118.1, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.1, pH 7.5) at 37°C. A bipolar platinum rings electrode was positioned around the tendinous portion of the muscle and field stimulation was done using a Panlab® LE12406TC stimulator (0.1Hz, 0.2ms, 5-12V) and the myographical records were isometrically obtained via MLT0201 model force-displacement transducers coupled to software-controlled model FE224 DC bridge transducer amplifiers (all from ADInstruments). Data acquisition was done using a PowerLab 4/35 system containing a LabChart and LabChart Pro software (PL3504/P) connected to a LE124060M Power Unit (ADInstruments). The preparations were stabilized for at least 20 min before addition of 110 µM acetylcholine (ACh) or 20 mM potassium chloride (KCl) at the beginning and at the end of each experiment [5], (Figure 1). The muscle response to exogenous agonist is a contracture for as long as the depolarizing agent remains activating the receptors, while the amplitude of the tension response is related to the number of receptors occupied by the drug [10].

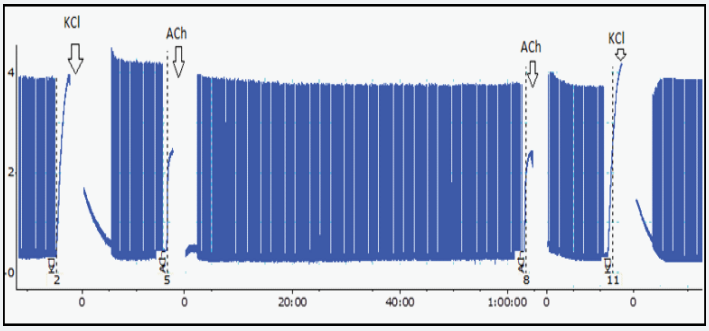

Figure 1: A typical myographic register from BC preparation [5], at two moments: under indirect stimulation (see twitches) and with absence of field stimulation (see contractures to ACh and KCl addition, before and after the experiment). Initial twitches mean the control, which amplitude represents 100%. Every 10 min the amplitude was measured and converted to percentage. Arrows, time addition (ACh or KCl). Tension, 1 g.

Experimental protocols

All experiments were carried out during 120 min and consisted of: Krebs control; *B. jararaca* venom (40 µg/mL, n=6), concentration-response curve of *Vh*-E (200, 400 and 600 µg/mL, n=5 each) resulting in the *Vh*-E concentration selection (600 µg/mL), preincubation (Pre-*vit,* n=4); and post venom (Post-*vit*, n=4) assays.

STATISTICAL ANALYSIS

The results were expressed as the mean±SEM. The number of experiments (n) is indicated in the legends of their respective figures. The results were analysed statistically using Student's *t*-test with the confidence level set at 5% ($p<0.05$).

RESULTS AND DISCUSSION

The medicinal properties of *Vochysia haenkeana* (Spreng.) Mart. (Vochysiae) have been related to antimicrobial activities and for treating respiratory diseases [11], with no toxicity to humans reported in the literature. Lima et al. [12], described the leishmanicidal activity of *Vh*-E against promastigotes of *Leishmania amazonenses* as being 85.1±0.058 µg/mL. In this work, we have assessed the ability of *V. haenkeana* extract (*Vh*-E) to neutralize the effects caused by *Bothrops jararaca* venom *in vitro* on chick biventer cervicis (BC) preparation. The venom of *B. jararaca* is known to induce severe local and systemic effects such as tissue damage, haemorrhage, coagulopathies, hypotension and renal cortical necrosis [13].

Figure 2 shows a concentration-response curve using 200, 400 and 600 µg/mL (n=5) of *Vh*-E, all tested concentrations there was no statistical difference compared to those preparations maintained in Krebs solution alone or among themselves. Based on these results, we select the major concentration of *Vh*-E (600 µg/mL) to be used under pre- and post-venom incubation treatments (Pre-*vit* and Post-*vit*). *B. jararaca* venom (40 µg/mL) induced 72.5±4.6% of blockade compared to control preparations after 120 min incubation ($p<0.05$, n=6). Zamunér et al. [27], using 50 µg/mL of *B. jararaca* venom had a time of 50% neuromuscular blockade (T50%) in 107.3±2.6 min, while the concentration of 40 µg/mL venom used in this work had T50% in 80 min, considered ideal for further neutralizing assays.

Vh-E under Pre-*vit* attenuated the neuromuscular blockade by *B. jararaca* venom (72.5±4.6% vs. 45.2±14% of blockade for venom alone and Pre-*vit*, respectively, $p<0.05$, n=4-6), however, the Post-*vit* was not able to avoid the neuromuscular blockade caused by *B. jararaca* venom (72.5±4.6% vs. 63.4±8.2% of blockade for venom alone and

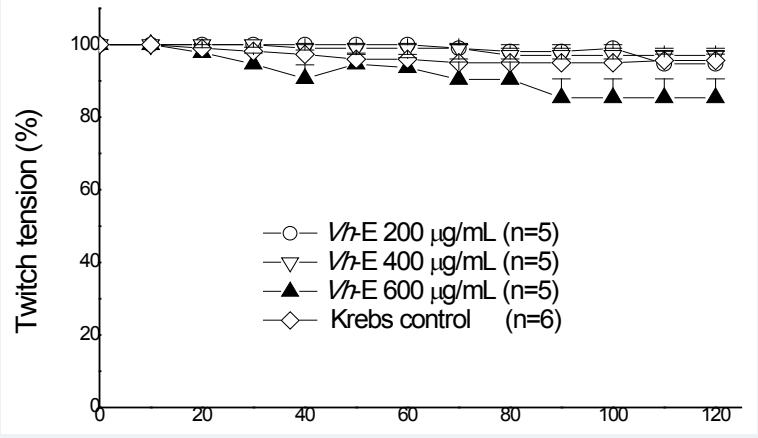

Figure 2: Chik biventer cervicis preparation, under field stimulation. All tested concentrations of *Vh*-E were statistically different from Krebs control, but not between themselves. Each point represents the mean±SEM of the number of experiments (n) showed in the legend. *Vh*-E, *Vochysia haenkeana* extract.

Post-*vit*, respectively, n=4-6) (Figure 3). The most studied antiophidian plants at the neuromuscular junction exhibits an *in vitro* ability in counteracting the neuromuscular blockade-induced by venoms, such as: *Casearia sylvestris* Sw. [14,15], *Plathymenia reticulata* Benth. [16], *Mikania laevigata* Sch. Bip. ex Baker [8,17], *Dipteryx alata* Vogel [18], *Camellia sinensis* L. [19,20], *Hypericum brasiliense* Choisy [21], *Vellozia flavicans* Mart. Ex Schult. [22], but few plants have been studied by its ability in counteracting the toxic effects after the start of venom action, like as *Casearia gossypiosperma* Briquet [23,24], *Jatropha elliptica* (Pohl) Oken. [25] and *Terminalia fagifolia* Mart. [22]. Post-*vit* assays allows to show the capacity of certain plants in reaching the local where the snake venom is and avoiding the paralysis evolution [25].

BC preparations with their multiple innervated fibers allow to induce contracture responses to exogenous ACh and KCl without electrical stimulation in order to assess the function of the post-synaptic receptors and muscle membrane integrity, respectively [26]. In those BC preparations incubated with *Vh*-E alone, there was no statistically change on the muscle contracture responses to exogenous ACh and KCl (Figure 4A), in addition, *B. jararaca* venom abolished both of contracture responses after 120 min incubation (Figure 4B). Envenomation by *B. jararaca* venom induce local and systemic myotoxicity and it has already been described its myotoxicity *in vitro* [10,27].

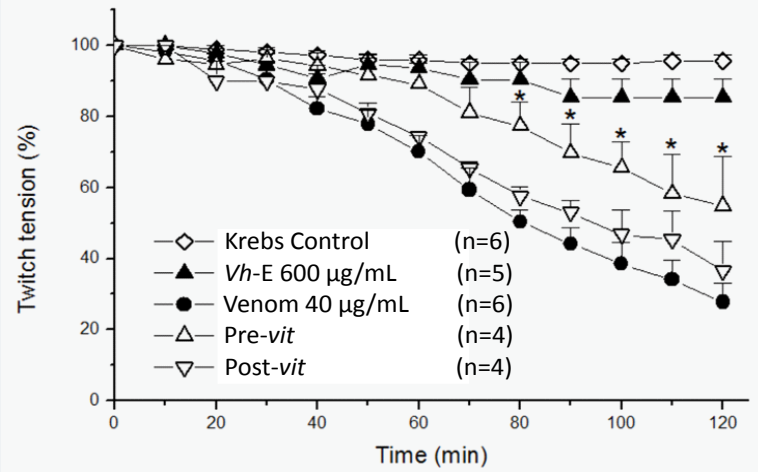

Figure 3: Chick biventer cervicis preparation, under field stimulation. All tested protocols were statistically different from Krebs control. Only Pre-*vit* protocol was statistically significant in comparison to venom (*p<0.05), with 54.8±14% functioning fibers at the end of experiment, while only 36.6±8.2% (p>0.05 when compared to venom) were active in the Post-*vit* protocol. Each point represents the mean±SEM of the number of experiments (n) showed in the legend. Venom, *Bothrops jararaca* venom. *Vh*-E, *Vochysia haenkeana* extract.

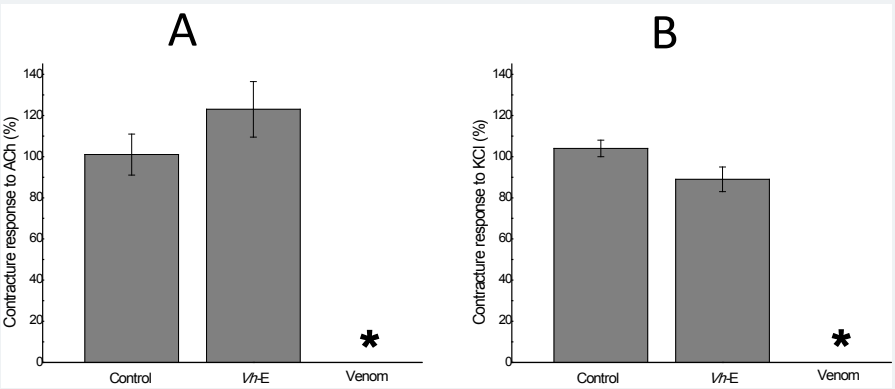

Figure 4: Chick biventer cervicis preparation, in absence of electrical stimulation. Exogenous ACh (A) and KCl (B) added at the end of experiment (120 min) show a responsive contracture of Krebs control of 101±10% and 104±4%, respectively (n=6). *V. haenkeana* extract (*Vh*-E) did not alter significantly these parameters, but notice the effect of the venom (*B. jararaca*) abolishing totally the contracture response of them (n=6). *p<0.05 compared to control.

Vh-E through Pre-*vit* was able to inhibit completely the effect of *B. jararaca* venom on the contracture response to exogenous ACh (106±17% of response, $p<0.05$), in addition, *Vh*-E under Post-*vit* attenuated the blockade induced by venom on the contracture to ACh (55±5% of response, $p<0.05$) (Figure 5A). *Vh*-E under both of treatments attenuated significantly the effect of *B. jararaca* venom on contracture responses to KCl (62.5±7.7% and 55±5% of response for Pre-*vit* and Post-*vit*, respectively; n=4), there was no significant difference between the treatments (Figure 5B).

When preincubated with the venom *Vh*-E also totally preserved the contracture responses to ACh. This effect constitutes evidence that this extract totally abolished the venom action on "extrinsic", but not on "intrinsic" nicotinic receptors (those elicited by indirect stimuli). Clearing, biventer cervicis muscle contains both focally (sensitive to electric stimulation resulting in twitch responses) and multiply (sensitive to agonist addition resulting in contracture responses) innervated fibers which, in turn, possess "intrinsic" and "extrinsic" nicotinic receptors, respectively [10,28]. According to Ginsborg [29], the nerve stimulation leads to contract the both types of muscle fibers, while exogenous ACh causes contraction of only the diffusely-innervated muscle fibers. It is believed that intrinsic receptors are less easily accessible than the extrinsic ones, due to a diffusion barrier in the narrow synaptic cleft. When the extract was added after the beginning of *B. jararaca* venom action, the protective effect of *Vh*-E was only of 55±5%.

Actions of drugs directly on muscle contractility can be assessed on preparations stimulated directly by addition of KCl or by electrical stimulation after abolition of neuromuscular transmission [10]. It is known that KCl causes muscle membrane depolarisation by releasing Ca^{2+} from its storage site in the sarcoplasmic reticulum (SR), which consequently activates the sarcomere [30]. In this study, we choose the first protocol. The effect of muscle fibers visualized by the contracture responses to exogenous KCl addition showed a partial protection exerted by *Vh*-E in both, preincubation and post venom treatments. At the same time shows the effect of the venom abolishing totally the contracture response to KCl and the level of venom influence on the muscle contractility. Besides, the biventer cervicis preparation was recommended by Harvey et al. [31], as a standard preparation for the screening of snake venoms for both, neurotoxic and myotoxic effects; and for checking that antivenom can neutralize such effects of venoms [32]. The measure of KCl contracture before and after the experiment is an indicator of muscle membrane integrity, which, in case of *Vh*-E showed to protect statistically against myotoxicity of *B. jararaca* venom.

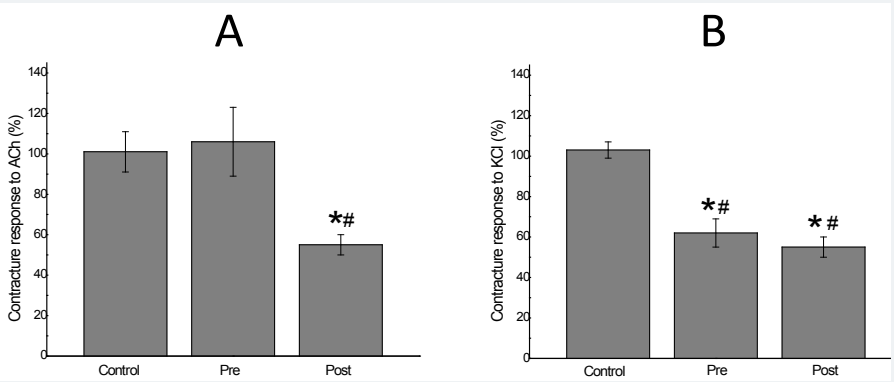

Figure 5: Chick biventer cervicis preparation, under no field stimulation. Exogenous ACh (A) and KCl (B) added at the end of experiment (120 min) show a responsive contracture of neutralization assays. *V. haenkeana* extract totally inhibited the venom effect in Pre (Pre-*venom incubation treatment*) to ACh addition, but partially against the Post (Post-*venom incubation treatment*). When KCl was added in post venom model, the responses of two models were similar.
* $p<0.05$ compared to control. # $p<0.05$ compared to venom.

CONCLUSION

The *Vh*-E neutralizes partially the neuromuscular blockade in Pre-*vit*, an effect that can be related to preserved function of "extrinsic" post-synaptic receptors, by measured contractures in response to ACh. The myotoxicity of the venom was significantly reduced by *Vh*-E in both, Pre-*vit* and Post-*vit*, by measured contractures in response to KCl.

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