

## RESEARCH REPORT

## Presynaptic effect of a methanolic extract of toad (*Rhinella schneideri*) poison in avian neuromuscular preparation

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Received: 27 May 2011; Accepted: 11 September 2011; Published: 22 September 2011

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### ABSTRACT

The neurotoxicity of a methanolic extract of toad (*Rhinella schneideri*) poison was examined in chick biventer cervicis preparations. The methanolic extract (1, 3, 10 and 30 µg/ml) caused concentration-dependent blockade at the three highest concentrations (time for 50% blockade, mean±SEM: 84±10, 51±3 and 12±0.8 min for 3, 10 and 30 µg/ml, respectively; n=6-8 each) that was preceded by significant, transient facilitation at 10 µg/ml. Contractures to exogenous ACh (110 µM) or KCl (20 mM) were unaffected by the blockade. In curarized (d-Tc, 1 µg/ml) preparations, the extract (10 µg/ml) caused complete, irreversible blockade that persisted after extensive washing. The extract did not significantly alter the creatine kinase release or morphology of biventer cervicis muscle. These results indicate that the methanolic extract of *R. schneideri* poison acts primarily presynaptically to enhance neurotransmitter release in this avian preparation.

**KEYWORDS:** Neuromuscular blockade, neurotransmitter release, presynaptic, *Rhinella schneideri*, toad poison

### INTRODUCTION

Toads have a highly toxic poison that is produced by well-developed, post-orbital parotid glands (Toledo et al, 1992). Toad poison contains a wide variety of compounds such as biogenic amines (Gregerman, 1952), peptides (O'Rourke et al, 2004), steroids and steroidal alkaloids (Zelnik, 1965) and proteins (Mahan and Biggers, 1977) with diverse biological properties, including enzyme (Zhao et al, 2005) and cancer (Meng et al, 2009) inhibition, antimicrobial activity (Tempone et al, 2008) and apoptotic activity (Qi et al, 2010). This poison may be used by toads in active or passive defense (Jared et al, 2009).

The cardiovascular effects of toad poison in vertebrates are well-known and have been extensively investigated, particularly in the genus *Rhinella* (Chen and Kovarikova, 1967; Sakate and Oliveira, 2000). In humans, envenoming with toad poison also produces a digoxin-like effect attributable to the presence of cardioactive glycosides (Gowda et al, 2003)

and this is the basis for treatment with digoxin-specific Fab antibody fragments (Brubacher et al, 1999). In contrast, little is known of the neuromuscular activity of toad poisons, although envenoming in dogs may be accompanied by neurological manifestations such as mydriasis, nystagmus and opisthotonus (Camplesi AC, 2006, MSc dissertation, Universidade Estadual Paulista – UNESP, Botucatu, SP, Brazil).

*Rhinella schneideri* (Frost, 2009), previously known as *Bufo paracnemis* (Lutz, 1925) is a common toad in some South American countries. In this work, we examined the neurotoxicity of a methanolic extract of Brazilian *R. schneideri* poison in avian neuromuscular preparations *in vitro*.

### MATERIALS AND METHODS

#### Reagents and venom

Acetylcholine (ACh) and *d*-tubocurarine were from Sigma Chemical Co (St Louis, MO, USA). Toad poison was

collected by manual compression of the large post-orbital parotid glands and a quantity of 2gm was then extracted with methanol (50ml) (Gao et al, 2010) for three days at room temperature, after which the resulting extract was lyophilized in a SpeedVac centrifuge. The methanolic extract was lyophilized and dissolved in Krebs solution prior to testing in the neuromuscular preparations.

### Animals

Male HY-LINE W36 chicks (4-8 days old) were supplied by Granja Globo Aves Agrovícola Ltd (Mogi Mirim, SP, Brazil). The chicks were housed at 25±3°C on a 12hr light/dark cycle with free access to food and water. This work was approved by an institutional Committee for Ethics in Animal Use (CEUA/UNICAMP, Protocol No. 1552-1) and was done in accordance with the ethical guidelines established by the Brazilian Society of Laboratory Animal Science (SBCAL).

### Chick biventer cervicis preparations

Biventer cervicis muscles obtained from male chicks (Ginsborg and Warriner, 1960) killed with isoflurane were mounted under a tension of 1gm/0.5cm in a 5ml organ bath containing warmed (37°C), aerated (95% O<sub>2</sub>+ 5% CO<sub>2</sub>, v/v) Krebs solution of the following composition: 118.7mM NaCl, 4.7mM KCl, 1.8mM CaCl<sub>2</sub>, 25mM NaHCO<sub>3</sub>, 1.17mM MgSO<sub>4</sub>, 1.17mM KH<sub>2</sub>PO<sub>4</sub> and 11.65mM glucose, pH 7.5. A bipolar platinum ring electrode was placed around the tendon within which runs the nerve trunk supplying the muscle. Field stimulation (0.1Hz, 0.2ms, 4-6V) was done with a Grass S48 stimulator (Astro-Med Inc, W Warwick, RI, USA). Muscle contractions and contractures were recorded isometrically via a force-displacement transducer (Load Cell BG- 10GM, Kulite Semiconductor Products Inc., NJ, USA) coupled to a Gould model RS3400 physiograph via a Gould universal amplifier (Gould Inc, Cleveland, OH, USA). Contractures to exogenously applied ACh (110µM) and KCl (20mM) were obtained in the absence of field stimulation prior to the addition of methanolic extract and at the end of the experiment, as a test for the presence of myotoxic and neurotoxic activities (Harvey et al, 1994). The preparations were allowed to stabilize for at least 20min before the addition of ACh, KCl or methanolic extract (1, 3, 10 and 30µg/ml) to the bath. Twitch-tension responses to the extract were monitored for up to 120min, depending on the extract concentration.

### Assessment of muscle fiber damage: creatine kinase release and histological analysis

Muscle damage by the methanolic extract was assessed by monitoring the increase in creatine kinase (CK) activity in the bathing solution and by histological analysis of chick biventer cervicis muscle tissue. For CK activity, 50µl aliquots of the bathing solution were withdrawn from the organ bath before (time zero) and after an 80min incubation with methanolic extract (10µg/ml) or Krebs solution alone (control). The samples were stored at 4°C and CK activity (expressed in U/l) was measured within 2hr of sample collection using commercial kits (CK-NAC BIOCLIN, Quibasa - Química Básica Ltda, Belo Horizonte, MG, Brazil).

At the end of the incubations, control and extract (10µg/ml)-treated biventer cervicis preparations were rapidly removed and fixed in 10% (v/v) formaldehyde for 24hr followed by dehydration in an ethanol series and embedding in paraffin. Sections 7-µm thick were stained with hematoxylin-eosin prior to examination and documentation with an Olympus BX51 photomicroscope fitted with an image capture system (Olympus Optical Co Ltd, Tokyo, Japan) and Image Pro-Plus 4.0 software (Media Cybernetics). Morphological damage was quantified by counting the number of fibers with lesions and expressing this as a percentage of the total number of fibers in three non-overlapping, non-adjacent areas of each muscle. This procedure was used in all experiments (control and treated preparations).

### Statistical analysis

Each experimental protocol was repeated 3-8 times and the results are reported as the mean ± SEM. Student's *t*-test and repeated-measures analysis of variance (ANOVA) were used for statistical comparison of the data, with a value of *P* < 0.05 indicating significance. All data analyses were done using OriginPro 8.

## RESULTS

### Neuromuscular activity of the methanolic extract

The methanolic extract (3-30µg/ml) caused time- and concentration-dependent neuromuscular blockade in chick biventer cervicis preparations that was preceded by a significant, transient facilitatory response (increase in muscle contractility) at a concentration of 10µg/ml; the lowest concentration of extract (1µg/ml) had no effect in these preparations (Figure 1A). The time for 50% neuromuscular blockade at 37°C was 84 ± 10min, 51 ± 3min and 12 ± 0.8min, for 3, 10 and 30µg/ml, respectively (n=6-8; *p* < 0.05 compared to each other). The neuromuscular blockade did not significantly affect the contractures to exogenous ACh and KCl (Figure 1B).

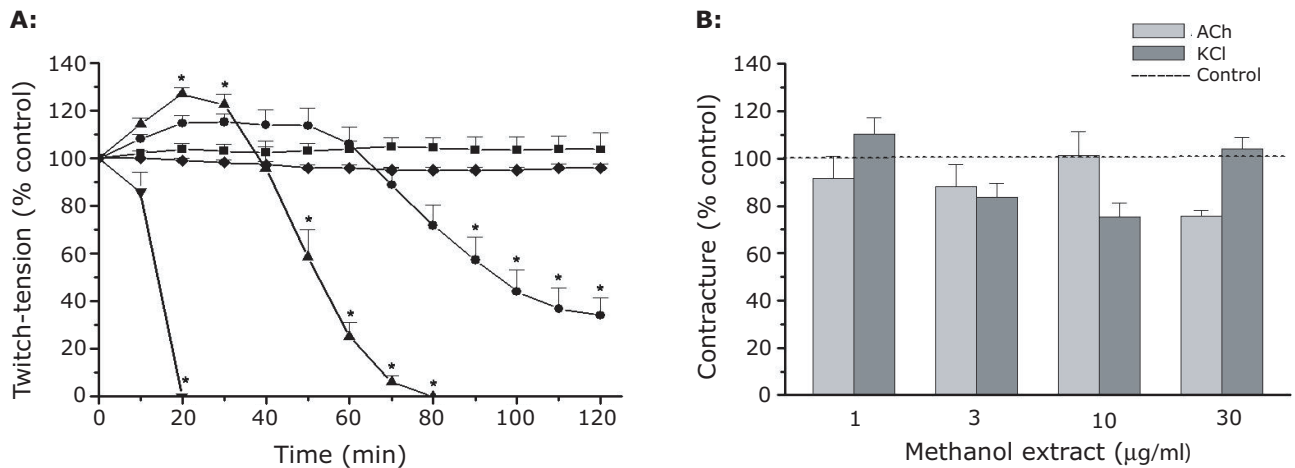
In biventer cervicis preparations pre-incubated with *d*-tubocurarine (*d*-Tc, 1µg/ml) alone for 70min (to block the response of pre- and postsynaptic nicotinic receptors to exogenous ACh) the neuromuscular blockade was reversible with washing (Figure 2A). In contrast, in preparations pretreated with *d*-Tc and then incubated with extract (10µg/ml), washing did not reverse the *d*-Tc-induced blockade (Figure 2B).

### Assessment of muscle fiber damage by the methanolic extract

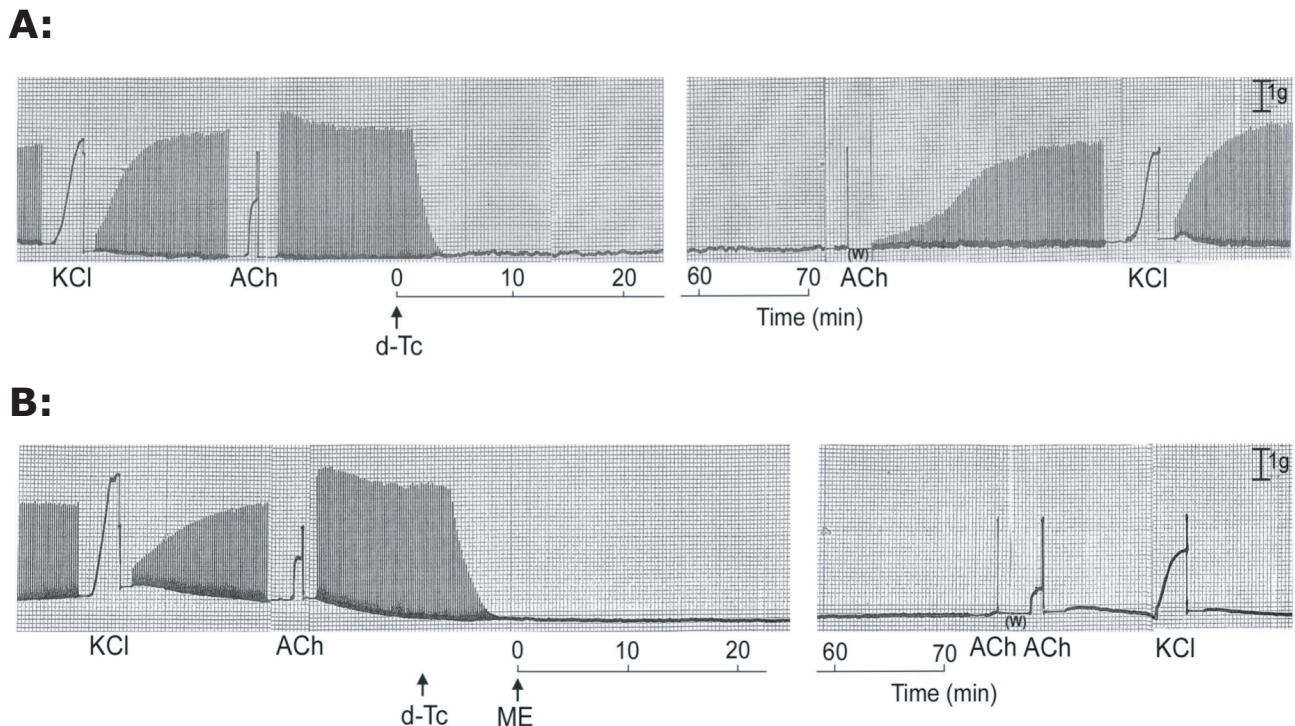
Incubation of biventer cervicis preparations with methanolic extract (10µg/ml) did not significantly alter the release of creatine kinase or the extent of muscle fiber damage after 80min (when complete neuromuscular blockade was observed) compared to control preparations (Table 1).

## DISCUSSION

Toad (*Rhinella* spp.) poison contain biogenic amines, peptides, steroids and steroidal alkaloids with a variety of biological activities, including cardiotoxicity, myotoxicity, neurotoxicity,



**Figure 1.** A. Neuromuscular blockade produced by a methanolic extract of *R. schneideri* poison in indirectly stimulated chick biventer cervicis preparations. B. Contractures to exogenous ACh (110µM) and KCl (20mM) in chick biventer cervicis preparations incubated with a methanolic extract of *R. schneideri* poison. The extract had no significant effect on the responses to exogenous ACh and KCl. The points represent the mean±SEM of 5-6 experiments. \* $p < 0.05$  compared to twitch-tension at time 0.



**Figure 2.** Neuromuscular responses to a methanolic extract of *R. schneideri* poison in biventer cervicis preparations pre-incubated with *d*-tubocurarine (*d*-Tc; 1µg/ml). Preparations were incubated with *d*-Tc alone (A) and with a methanolic extract of *R. schneideri* poison (ME, 10µg/ml) after neuromuscular blockade induced by *d*-Tc (B). Note that washing (W) restored the twitch-tension in preparations incubated with *d*-Tc alone but had no effect in preparations incubated with toad poison after *d*-Tc-induced blockade. Contractures to exogenous ACh (110µM) and KCl (20mM) were obtained before and after incubation with *d*-Tc or *d*-Tc and extract. Note the restoration of the contracture to ACh in (B) after washing (w). These recordings are representative of four additional experiments.

**Table 1.** Muscle fiber parameters after incubation with a methanolic extract (10 g/ml) of toad (*R. schneideri*) poison. The values are the mean ± SEM of the number of experiments (n) indicated. There were no significant differences between the results for control preparations (incubated with physiological solution alone) and those treated with extract. The proportion of damaged fibers and CK release were assessed after 80 min, when complete inhibition was observed with the extract.

Treatment (n)	Damaged fibers (%)	CK release (U/l)
Control (4)	2.4 ± 0.9	80 ± 15
Methanolic extract (5)	2.3 ± 0.5	90 ± 21

vasoconstriction and hypotension, as well as one of the most potent hallucinogens known, 0-methylbufotenine (Daly and Witkop, 1966). The results described here clearly show that a methanolic extract of *R. schneideri* poison contains substances capable of affecting avian neurotransmission.

In chick biventer cervicis preparations, the methanolic extract (3-30µg/ml) caused concentration-dependent neuromuscular blockade that was preceded by significant facilitation of neurotransmission at 10µg/ml. The transient duration (~10-15min) of this facilitation most likely reflected attenuation of this response by the onset of neuromuscular blockade. Although some facilitation was observed with an extract concentration of 3µg/ml the increase was not significantly different from control preparations. No facilitation was seen with an extract concentration of 30µg/ml, probably because the onset of neuromuscular blockade was so rapid that it masked any facilitation. Facilitation in neuromuscular preparations generally reflects enhanced presynaptic release of ACh, and neuromuscular blockade preceded by facilitation is commonly observed with presynaptically active venoms and/or toxins, e.g., β-bungarotoxin, notexin (Su and Chang, 1984) and crotoxin (Hawgood and Smith, 1989).

The blockade observed here was biphasic, with an increase in twitch-tension followed by complete neuromuscular blockade; a similar biphasic response has also been observed in mammalian preparations incubated with snake venom presynaptic neurotoxins such as β-bungarotoxin, notexin (Su and Chang, 1984) and crotoxin (Hawgood and Smith, 1989). However, the biphasic response seen here was not accompanied by alterations in the muscle contractures to exogenous ACh or KCl. These findings indicate that the extract had no inhibitory effect on postsynaptic nicotinic receptors and also did not interfere with the muscle contracture mechanism. Neuromuscular blockade with these characteristics has been attributed to an inhibitory presynaptic action (Harvey et al, 1994; Lewis and Gutman, 2004).

The experiments with *d*-Tc, which reversibly prevents twitch tension responses and blocks the responses of pre- and postsynaptic nicotinic receptors to exogenous ACh (Webb and Bowman, 1974), showed that the methanolic extract abolished the recovery of twitch responses normally seen with *d*-Tc alone after washing, but did not adversely affect the contractures to exogenous ACh and KCl (compare Figure 2B with 2A). This finding indicated that the extract did not affect post-synaptic nicotinic receptors or muscle excitability since the responses to ACh and KCl, respectively, were preserved. Consequently, a presynaptic action must account for the ensuing loss of twitch responses. The observation that the contractures to exogenous ACh and KCl, both of which are postsynaptic responses, were unaltered by the extract also indicates that the blockade did not involve a direct effect on the postsynaptic muscle action potential.

Taken together, these results indicate that the methanolic extract of *R. schneideri* poison causes neuromuscular blockade by a presynaptic action in chick biventer cervicis preparations, without damaging muscle fibers.

## ACKNOWLEDGMENTS

We thank Marta Beatriz Leonardo, Geraldino Cunha Filho and Aracélio Viana Colares for technical assistance. This work is part of a PhD thesis by S.R.F. presented to the Programa de Pós-Graduação em Farmacologia (Faculdade de Ciências Médicas, UNICAMP) and was supported by a studentship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil). L.R.S., M.A.C.H. and S.H. are supported by research fellowships from CNPq.

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