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Antibothropic Action of *Camellia sinensis* Extract Against the Neuromuscular Blockade by *Bothrops jararacussu* Snake Venom and Its Main Toxin, Bothropstoxin-I

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1. Introduction

Snake bite envenoming, a serious public health problem in rural areas of tropical and subtropical countries, was included in 2007 as a neglected disease by the World Health Organization (WHO, 2007). Under this geographical perspective Africa, Asia, Oceania and Latin America are the most vulnerable countries to this kind of accident, but also shared by many developing countries (Harrison et al., 2009; Warrel, 2010). An excellent meta-analytic approach about the subject was described by Chippaux (2011), who analysed more than 3,000 references for estimating the burden of snakebites in sub-Saharan Africa. Brazil encloses both requirements, as a developing and a tropical country, and needs to strengthen measures against venomous snake accidents, since, according to Lima et al. (2009), it is the country with the major number of accidents (about 20,000 cases/year), followed by Peru (4,500), Venezuela (2,500-3,000), Colombia (2,675), Ecuador (1,200-1,400) and Argentina (1,150-1,250) (Warrel, 2004).

As mentioned by Nicoleti et al. (2010), venomous snakes in Brazil are represented by *Bothrops*, *Bothropoides*, *Bothriopsis*, *Bothrocophias*, *Rhinocrophis*, *Crotalus*, *Lachesis*, *Leptomicrurus* and *Micrurus* (see the new taxonomic arrangement proposed by Fenwick et al., 2009). Envenoming by the first five genera produce similar toxic manifestations and treatment assessment are quite the same. They represent 86.9% of accidents, whereas 8.7% were caused by *Crotalus*, 3.6% *Lachesis* and 0.8% by *Leptomicrurus* and *Micrurus* (Ministério da Saúde, 2004).

Bothrops jararacussu snake belongs to the Viperidae family and its venom is able to induce severe signs of local and systemic envenoming, such as necrosis, shock, spontaneous

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systemic bleeding and renal failure, incoagulable blood and death (Milani et al., 1997); its venom also blocks *in vitro* the contractile skeletal muscle response (Rodrigues-Simioni et al., 1983). Two myotoxins are responsible for myonecrosis: bothropstoxin-I (Homsí-Brandeburgo et al., 1988), the first myotoxin isolated from the venom that reproduces the effects of the crude venom (Heluany et al., 1992), further characterized as a phospholipase A₂-Lys49 (Cintra et al., 1993); and bothropstoxin-II, a phospholipase A₂ (Gutiérrez et al., 1991), further characterized as an Asp49-PLA₂ myotoxin with low catalytic activity (Pereira et al., 1998), although phylogenetically it is more related to Lys49-PLA₂s than to other Asp49-PLA₂s (dos Santos et al., 2011).

Snake antivenom immunoglobulins (antivenoms) are the only specific treatment for envenoming by snakebites. They are produced by fractionation of plasma usually obtained from large domestic animals hyper-immunized against relevant snake venoms. When injected into an envenomed human patient, antivenom will neutralize any of the effects of the venoms used in its production, and in some instances will also neutralize effects of venoms from closely related species (WHO, 2011a). However, the antithrotoppic serum effectiveness against the local effects of *Bothrops jararacussu* venom (one of the bothropic venoms used in the serum production) has been debated since the 80's decade (see Correa-Neto et al., 2010). A possible explanation for the lack of effectiveness was given by Battellino et al. (2003) through the use of intravital microscopy after intravenous administration of antithrotoppic antivenom (BAv), labeled with fluorescein isothiocyanate (FITC). They observed that the antivenom neutralized the systemic effects, but did not efficiently reverse the local effects due to an impaired and/or delayed venom:antivenom interaction at the site of injury. Considering that local effect of venomous snakebites are poorly prevented by specific antivenom, that the access to public health services by people of distant rural regions in tropical and subtropical countries is in general difficult, the use of medicinal plants as a local solution has been a practice of natives of those regions.

Medicinal plants represent a sophisticated biotechnological laboratory that is able to produce a multitude of pharmacologically bioactive substances, with a wide variety of effects (Mahmood et al., 2005). The second beverage (next to water) of major consumption in the world, in its green, black and oolong forms, is the tea from *Camellia sinensis* L. leaves. Compounds as polyphenols, polysaccharides, aminoacids, vitamins (Crespy & Williamson, 2004), caffeine and a very small amount of methylxanthines (Yang et al., 1998) can be found in the plant. Catechins, the major component of green tea (fresh leaves are steamed to prevent fermentation, yielding a dry, stable product), and represent the low-molecular-weight polyphenols consisting mainly of flavanol (flavan-3-ol) monomers, such as epicatechin, epicatechin-3-gallate, epigallocatechin and the major, 50-80% of the total catechin, epigallocatechin-3-gallate (Graham, 1992; Khan & Mukhtar, 2007). Catechins account for 6-16% (Zhu & Chen, 1999) up to 30-40% (Phithayanukul et al., 2010) of the dry green tea leaves. The fermentation or semifermentation stage (when the withered leaves are rolled and crushed) during the manufacture of black or oolong tea, respectively, converts catechins to theaflavins (theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3,3'-digallate, accounting for 3-6% of solid extract) and thearubigins (accounting for 12-18% of solid extract) (Leung et al., 2001; Khan & Mukhtar, 2007), which are complex polyphenols of poorly-defined chemical structures formed during fermentation or polymerization of theaflavins (Hazarika et al., 1984).

The literature describing the medicinal benefits of tea is extensive, but the report about its consumption to alleviate post game fatigue in players and sportsmen (Krishnamoorthy, 1991) inspired further studies on the mammalian skeletomotor apparatus (Das et al., 1994; 1997). For example, Basu et al. (2005) attributed to theaflavin, but not thearubigin, the facilitatory effect induced at the skeletal myoneural junction. This experimental model has been traditionally used for the pharmacological characterization of snake venoms, and the association between *C. sinensis* and snake venoms was a natural consequence. Thus, results showing the inhibitory effect of tea polyphenols on local tissue damage induced by snake venoms (Pithayanukul et al., 2010), and the inhibitory effect of *Camellia sinensis* leaves extracts against the neuromuscular blockade of *Crotalus durissus terrificus* venom (de Jesus Reis Rosa et al., 2010) were recently published. Here, using the same experimental procedure, the antivenom property of *Camellia sinensis* leaves extract was assayed against *Bothrops jararacussu* venom and its main myotoxin, bothropstoxin-I. Commercial theaflavin (from black tea) and epigallocatechin gallate (from green tea), known to be part of the *C. sinensis* extract, were also tested.

2. Materials and methods

2.1 Hydroalcoholic extract from leaves of *Camellia sinensis*

The leaves of *C. sinensis* were harvested from plants growing in an orchard at the University of Sorocaba – UNISO (Sorocaba, SP, Brazil). A voucher specimen was deposited in the Instituto Agrônomo de Campinas (IAC, number 50.469) herbarium (<http://herbario.iac.sp.gov.br>) after identification by L.C. Bernacci. Briefly, sixty-four grams of leaves powder were macerated along with 150 mL of 70° GL ethanol, over 3 days. After this period, the resulting suspension was placed into a percolator with 50 mL of 70° GL ethanol, and left for a further 3 days. The macerated drug was percolated and a 20% hydroalcoholic extract was obtained (de Jesus Reis Rosa et al., 2010). The solvent was evaporated until dryness, and the dried extract was then protected from light and humidity at room temperature until the assays.

2.2 Pharmacological study

2.2.1 Animals

Male Swiss white mice (26-32 g) were supplied by the Anilab - Animais de Laboratório (Paulínia, São Paulo, Brazil). The animals were housed at $25 \pm 3^\circ\text{C}$ on a 12-h light/dark cycle with access to food and water *ad libitum*. This study was approved (protocol number A077/CEP2007) by the Committee for Ethics in Research from the University of Vale do Paraíba (UNIVAP) and all experiments were performed according to the guidelines of the Brazilian College for Animal Experimentation.

2.2.2 Venom and toxin

The crude venom was obtained from adult *Bothrops jararacussu* (Bjssu) snakes (Serpentário do Centro de Estudos da Natureza) and certified by Prof. Dr. Jose Carlos Cogo, University of Vale do Paraíba (Univap), São Jose dos Campos, SP, Brazil. Bothropstoxin-I (BthTX-I) was obtained under the conditions described by Homsí-Brandeburgo et al. (1988).

2.2.3 Mouse phrenic-nerve diaphragm muscle (PND) preparation

The PND was obtained from mice anesthetized with halothane and sacrificed by exsanguination. The diaphragm was removed (Bülbring, 1946) and mounted under a tension of 5 g in a 5 mL organ bath containing continuous-aerated Tyrode solution (control) with the following composition: 137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 0.49 mM MgCl₂, 0.42 mM NaH₂PO₄, 11.9 mM NaHCO₃, and 11.1 mM glucose. After stabilization with 95% O₂/5% CO₂, the pH was 7.0. The PND myographic recording was performed according to Melo et al. (2009). Briefly, preparations were stimulated indirectly with supramaximal stimuli (4 x threshold, 0.06 Hz, 0.2 ms) delivered from a stimulator (model ESF-15D, Ribeirão Preto, SP, Brazil) to the nerve through bipolar electrodes. Isometric twitch tension was recorded with a force displacement transducer (cat. 7003, Ugo Basile), coupled to a 2-Channel Recorder Gemini physiograph (cat. 7070, Ugo Basile) via a Basic Preamplifier (cat. 7080, Ugo Basile). PND was allowed to stabilize for at least 20 min before addition of the following substances: BthTX-I alone at 20 µg/mL (n=11); Bjssu alone at 40 µg/mL (n=5); 20 µg/mL BthTX-I + 0.05 mg/mL *C. sinensis* extract (n=5); 40 µg/mL Bjssu + 0.05 mg/mL *C. sinensis* extract (n=3); 40 µg/mL Bjssu + 0.025 mg/mL epigallocatechin gallate (n=3, Sigma-Aldrich, SP, Brazil); 40 µg/mL Bjssu + 0.05 mg/mL theaflavin (n=3); and the controls nutritive Tyrode solution (n=7) and 0.05 mg/mL *C. sinensis* extracts (n=7). The plant extract or commercial phytochemicals concentrations were chosen based on the minor changes obtained in comparison with the basal response of PND incubated with Tyrode nutritive solution (control).

2.3 Quantitative histological study

At least three preparations (n=3) resulting from pharmacological assays were analyzed by quantitative morfometry. Preparations used in the controls, nutritive Tyrode solution and *C. sinensis* hydroalcoholic extract (0.05 mg/mL) were compared to BthTX-I (20 µg/mL), or *C. sinensis* (0.05 mg/mL) + BthTX-I (20 µg/mL) groups, or Bjssu (40 µg/mL), or *C. sinensis* (0.05 mg/mL) + Bjssu venom (40 µg/mL) after fixation in Bouin solution and submission to routinely morphological techniques. Cross-sections (5 µm thick) of diaphragm muscle embedded in paraffin were stained with Hematoxylin-Eosin for microscopy examination. Tissue damage was expressed in percentage (number of damaged muscle cells divided by the total number of cells in three non-overlapping, non-adjacent areas of each preparation) according to Cintra-Francischinelli et al. (2008).

2.4 Thin layer chromatography (TLC)

Aliquots of *C. sinensis* hydroalcoholic extract were spotted onto 0.2 mm thickness silica gel 60F₂₅₄ on aluminum plates, 20.10 cm, (Merck, Germany) and developed with ethyl acetate:methanol:water (100:13.5:10, v/v) in a pre-saturated chromatographic chamber along with appropriate phytochemical standards (Simões et al., 2004). These standards (theaflavin and epigallocatechin gallate, Sigma-Aldrich® - USA) were solubilized in methanol (1 mg/mL). The separated spots were visualized (under UV light at 360 nm) with NP/PEG as follows: 5% (v/v) ethanolic NP (diphenylboric acid 2-aminoethyl ester, Sigma Chemical Co., St. Louis, MO, USA) followed by 5% (v/v) ethanolic PEG 4000 (polyethylene glycol 4000, Synth Chemical Co., São Paulo, SP, Brazil). The retention factor (R_f) of each standard was compared with spots exhibited by *C. sinensis* extracts.

2.5 Statistical analysis

Each pharmacological protocol was repeated at least three times. Results were expressed as the mean ± standard error of the mean (SEM). The Student’s *t*-test or repeated measures ANOVA were used for statistical comparison of the data. The significance level was set at 5%.

3. Results

3.1 Pharmacological assays

3.1.1 BthTX-I neutralization

Figure 1 shows the PND blockade activity of BthTX-I (20 µg/mL, n=11), which was irreversible even after washing (W) of preparations with fresh nutritive Tyrode solution. However, the previous incubation of the toxin with 0.05 mg/mL *Camellia sinensis* extract totally (100%) prevented the characteristic neurotransmission blockade, showing a better functional outcome of neuromuscular preparation after washing. The 0.05 mg/mL of *Camellia sinensis* extract was chosen in all protocols since it induced minor changes compared with the basal response of PND.

Fig. 1. Isolated mouse phrenic nerve-diaphragm preparations under indirect stimuli. Note the total efficacy of *C. sinensis* extract in protecting the neuromuscular blockade induced by BthTX-I. Each point represents the mean ± SEM. * = *p*<0.05 in comparison with the bothropstoxin-I (BthTX-I); W, washing.

3.1.2 Bjssu neutralization

Figure 2 shows the PND blockade activity by Bjssu crude venom. There was no contraction recovery of PND after washing the preparation. *C. sinensis* extract was 78 ± 12 % able to neutralize the venom that, in turn, differently of its myotoxin, contains several constituents.

Fig. 2. Isolated mouse phrenic nerve-diaphragm preparations under indirect stimuli. Note the partial efficacy of *C. sinensis* extract in protecting the neuromuscular blockade induced by Bjssu. Each point represents the mean $\pm$ SEM. * = $p < 0.05$ in comparison with the venom. W, washing. Bjssu, *Bothrops jararacussu* venom.

3.2 Quantitative histological study

Figure 3 shows neuromuscular preparations exposed either to Tyrode (Fig. 3A) or *C. sinensis* extract (Fig. 3B): the muscle fibers were well-preserved, showing changes not significantly different between each other of 15.9 ± 0.8 % or 25.3 ± 1.1 % damaged fibers, respectively. These changes were related mainly to loss of the typical cell cross-sectional polygonal profile. Differently, BthTX-I (Fig. 3C, 66.6 ± 2.3 %) and venom (Fig. 3E, 75.1 ± 1.1 %) alone clearly showed in transversal sections characteristic signals of myonecrosis (m), edematous cells (e), loss of polygonal profile, sarcolemma disruption, delta lesion (arrow), “ghost” cells (g), and nuclei (n) dispersed in the tissue. These changes were already extensively described in the scientific literature. Panel 3D and 3F show cross-sections of PND muscle fibers after *in vitro* neutralization by *C. sinensis* extract of BthTX-I (23.4 ± 1.3 % of lesioned fibers, $p < 0.05$) and of Bjssu (27.8 ± 0.9 % of lesioned fibers, $p < 0.05$), respectively.

Fig. 3. Cross-sections (5 µm thick) of diaphragm embedded in paraffin and stained with Hematoxylin-Eosin. (A) Control-sham diaphragm preparation ($15.9 \pm 0.8 \%$). (B) Neuromuscular preparation exposed to 0.05 mg/mL *Camellia sinensis* extract ($25.3 \pm 1.1 \%$). (C) Muscle incubated with 20 µg/mL BthTX-I ($66.6 \pm 2.3 \%$). (E) Muscle incubated with 40 µg/mL Bjsu venom ($75.1 \pm 1.1 \%$). The main fibers damage are lettered as follows: myonecrosis (m), edema (e), delta lesion (arrow), sarcolemmal disruption with nuclei (n) dispersion, “ghost” cells (g) visualized by spaces optically empty. Note that area with extensive myonecrosis has a hyaline aspect. Muscles incubated with 0.05 mg/mL *Camellia sinensis* extract (D and F) shows fibers maintaining its characteristic polygonal profile in despite of a number of them being edematous (e). A slow percentage of them • $23.4 \pm 1.3 \%$ for BthTX-I (D); $27.8 \pm 0.9 \%$ for Bjsu (F) • • showed myonecrosis (m). Bars = 50 µm.

3.3 Efficacy of commercial phytochemicals against Bjssu venom

Figure 4 shows the effect of commercial 0.05 mg/mL theaflavin and 0.025 mg/mL epigallocatechin gallate from *C. sinensis* on twitch blockade induced by 40 µg/mL Bjssu venom. This paralysis was completely blocked (n=3, *p<0.05 compared to the venom, but did not show statistical differences with *C. sinensis* extract). In addition, following washing out of treated preparation with fresh physiological salt solution, twitch height was re-established (not shown).

Fig. 4. Isolated mouse phrenic nerve-diaphragm preparations under indirect stimuli. Antibothropic action of commercial phytochemicals from *Camellia sinensis*. Note total protection against the paralysis of Bjssu (*Bothrops jararacussu*) venom. Each point represents the mean ± SEM. * = p<0.05 in comparison with the crude venom.

3.4 Thin layer chromatography (TLC)

Figure 5 shows a chromatoplaque of *C. sinensis* leaves extract obtained by TLC exhibiting a complex variety of compounds including theaflavin and epigallocatechin as confirmed by R_f of these commercial phytochemicals. Panel A is the chromatoplaque exposed only to a UV light at 360 nm, whereas Panel B is the same plaque after NP/PEG chromogenic agent pulverization.

Fig. 5. Thin Layer Chromatography performed by using ethyl acetate:methanol:water (100:13.5:10) solvent/Developer: NP/PEG. Phytochemical standards: 1 - Epigallocatechin gallate ($R_f=0.80$); 2 - Cs, *Camellia sinensis* leaves extract; 3 - Theaflavin ($R_f=0.56$). Panel A: chromatoplaque exposed to UV light at 360 nm. Panel B: is the same plaque after NP/PEG chromogenic agent pulverization. Cs spots are suggestive of several flavonoids (yellow/orange fluorescence) and phenolic constituents (blue fluorescence), including epigallocatechin gallate and theaflavin, respectively. Comparative R_f values between phytochemicals and extract are highlighted in the circle. R_f , retention factor.

4. Discussion

Although the only specific treatment for envenoming by snakebites is immunoglobulins (antivenoms), since it can prevent or reverse most of the systemic effects and hence minimizing mortality and morbidity (WHO, 2011a), any alternative strategy aiming to interrupt or neutralize the steps of envenoming process can be effective for snakebite local effects. The clinical features of the bites of venomous snakes reflect the effects of these venom components that vary between species to species, but can broadly be divided into categories which include i) cytotoxins, causing local swelling and tissue damage, ii) haemorrhagins, which disturb the integrity of blood vessels, iii) compounds, which lead to incoagulable blood, iv) neurotoxins, causing neurotoxicity and iv) myotoxins, which cause muscle breakdown (WHO, 2011b). *Bothrops jararacussu* venom encloses all of them, except *in vivo* neurotoxicity (Milani et al., 1997), but it causes an *in vitro* neuromuscular blockade (Rodrigues-Simioni et al., 1983).

As snake accidents occur by bites and venoms are commonly injected in the subcutaneous muscle tissue, the use of muscle preparations as model for the study of the pharmacological effects of snake venom and toxins is very relevant. Besides, the use of snake venom and toxins as tools to study neuromuscular blockade *in vitro* (Gallacci & Cavalcante, 2010) is very useful given the excitation-contraction coupling process starts with transmission of electrical impulses from nerves towards muscle fibers via release of acetylcholine (ACh) (Hughes et al., 2006).

On the other hand, the plant kingdom represents a rich resource of new molecules able to counteract the venom effects, mainly when the plant is as worldwide as *Camellia sinensis*, an evergreen Asiatic shrub of the Theaceae family. Polyphenols from black or green tea has been shown to be powerful antioxidants with a potent inhibitory effect on low density lipoprotein (LDL) oxidation *in vitro* (Miura et al., 2000), exert anti-carcinogenic (Lambert & Yang, 2003) and anti-inflammatory (Arab & Il'yasova, 2003) effects; act as antibacterial and antiviral agents (Friedman, 2007), and are able to reduce the incidence of coronary heart disease and diabetes (Crozier et al., 2009), among other effects (see Khan & Mukhtar, 2007). Despite its health benefits, there are few studies using *C. sinensis* addressed to snake venom.

Hung et al. (2004) showed an antagonistic effect of 3 mg per mouse of melanin extracted from black tea (MEBT), an unhydrolyzed complex of tea polyphenols (Sava et al., 2001), against *Agkistrodon contortrix laticinctus* (broadbanded copperhead), *Agkistrodon halys blomhoffii* (Japanese mamushi), and *Crotalus atrox* (western diamondback rattlesnake) snake venoms, when administered i.p. immediately after venom administration in the same place of venom injection. Authors demonstrated correlation between antivenom activity of melanin and PLA₂ inhibition as a possible explanation for the protective effect.

Tea polyphenols have been shown to interact with hydrolytic enzymes from *Naja naja kaouthia* Lesson (Elapidae) and *Calloselasma rhodostoma* Kuhl (Viperidae) venoms, inhibiting inflammation and local tissue damage. This effect was attributed to complexation and chelation among the venom proteins and the phenolic contents of the extract. According to the authors, the *Camellia sinensis* extract also inhibited phospholipase A₂, proteases, hyaluronidase and L-amino acid oxidase by *in vitro* neutralization and the hemorrhagic and the dermonecrotic activities of the venoms *in vivo* (Pithayanukul et al., 2010).

Satoh et al. (2002 a,b) reported the protective effect of thearubigin from black tea extract against the neuromuscular blockade caused by botulin neurotoxins and tetanus toxin in synaptosomal membrane preparations. Recently, de Jesus Reis Rosa et al. (2010) reiterate the protective effect of *C. sinensis* leaves extracts which prevented *in vitro* the irreversible neuromuscular blockade typical of *Crotalus durissus terrificus* venom, more specifically caused by crotoxin, the main component of the crude venom (Slotta & Fraenkel-Conrat, 1983). We suggest that the target for *C. sinensis* protective effect is the motor nerve terminal, since the blockade caused by crotoxin, botulin toxin and tetanus toxin occurs by the inhibition of the neurotransmitter release, differently, from motor nerve terminals (Habermann et al., 1980).

Based on research findings suggesting an effective anti-cancer property attributed mainly to epigallocatechin-3-gallate (Fig. 6A) found primarily in green tea, and theaflavin (Fig. 6B) from black tea, both equally effective antioxidants (Leung et al., 2001), these two compounds were also assayed against *Crotalus durissus terrificus* venom (de Jesus Reis Rosa et al., 2010). Curiously, commercial theaflavin, but not epigallocatechin gallate, maintained

partial muscular activity in the presence of 5 µg/mL venom. Coincidentally, Basu et al. (2005) showed that only the theaflavin fraction from black tea was able to produce a facilitatory effect at the skeletomotor site, being this facilitation modulated by calcium and nitric oxide signaling.

Concerning the modulation of synaptic nerve-muscle interaction, it was found that ACh and glutamate are co-released from synaptosomes of *Torpedo electric* organ (Vyas & Bradford, 1987), also demonstrated in rat motor nerve terminals (Waerhaug & Ottersen, 1993). Glutamatergic receptors such as N-methyl-D-aspartate (NMDA) have been identified at the postsynaptic membrane in neuromuscular junction of adult rats (Urazaev et al., 1998; Grozdanovic & Gossrau, 1998). Glutamate released from nerve endings probably activates NMDA-receptor mediated Ca^{2+} entry into the sarcoplasm followed by activation of NO (Urazaev et al., 1998). Nonquantal ACh acting through M1-cholinergic receptors (Urazaev et al., 2000; Malomouzh et al., 2007), activates synthesis of NO to serve as a trophic message from motoneurons that keeps the Cl^- transport inactive in the innervated sarcolemma (Urazaev et al., 1999). For a better understanding of the synaptic nerve-muscle modulation, see also the study of Rubem-Mauro et al. (2009) that corroborates the nitric oxide role at the neuromuscular junction.

Fig. 6. Structures of major components of *Camellia sinensis*. A, epigallocatechin gallate (Zhu et al., 2008). B, theaflavin (Khan & Mukhtar, 2007).

The well-successful experience between *C. sinensis* leaf extract and presynaptic neurotoxins, and while bothropstoxin-I (BthTX-I) isolated from *Bothrops jararacussu* venom exhibits an earlier presynaptic action (Oshima-Franco et al., 2004) before its well-known myotoxic effect, the same experimental procedure was carried out using *C. sinensis* extract, which protected 100% the neuromuscular blockade (Fig. 1).

Different mechanisms have been proposed for BthTX-I myotoxic effect such as altering the bilayer membrane integrity (Lomonte et al., 2003), binding to the Ca^{2+} -binding region in the pore of Ca^{2+} channels (Oshima-Franco et al., 2004), activating membrane acceptors (Cintra-Francischinelli et al., 2009) or causing a general membrane-destabilizing (Gallacci & Cavalcante, 2009). In order to explain the rationale of this study more details will be given about these mechanisms.

BthTX-I represents a distinct group of PLA₂ homologue myotoxins containing Lys49 instead of Asp49 residue, with consequent loss of Ca²⁺-binding and enzymatic activity, the segment 115-129 of the C-terminal region, which includes a variable combination of positively charged and hydrophobic/aromatic residues, has the ability to alter the bilayer membrane integrity (Lomonte et al., 2003), a possible way by which *C. sinensis* extract could exert its protection against the toxic effect of BthTX-I.

Oshima-Franco et al. (2004) have shown that BthTX-I, at a concentration that does not produce neuromuscular blockade (0.35 mM) caused the appearance of giant miniature endplate potentials, without affecting the resting membrane potential. The authors suggested that the toxin would act through Ca²⁺ channels, since Mn²⁺ antagonized both neurotoxic and myotoxic actions of the myotoxin and are related to Ca²⁺ fluxes. Mn²⁺ is thought to bind to the Ca²⁺ -binding region in the pore of Ca²⁺ channels, thereby preventing the passage of calcium ions (Nachshen, 1984). The influence of the earlier presynaptic action of BthTX-I is relevant from the pharmacological point of view, as shown here using *C. sinensis* leaves extract, although clinically the bothropic envenomation shows no signs of neurotoxicity. However, *C. sinensis* extract also protected against the myotoxic effects of BthTX-I (Fig. 3D), showing a parallelism between neurotoxic and myotoxic effects of the myotoxin.

Cintra-Francischinelli et al. (2009) excluded the possibility that the inactive Lys49 toxins act by binding to a membrane channel, thus increasing its permeability to Ca²⁺. The authors have shown that the action of myotoxins from snake venoms on muscle cells begins with the activation of membrane acceptors coupled to intracellular Ca²⁺ stores, which is rapidly followed by the toxin dependent alteration of membrane permeability to ions (and other molecules). By this mechanism, *C. sinensis* is able to inactivate the acceptors signalization.

Gallacci & Cavalcante (2010) proposed a hypothetical mechanism for the *in vitro* neuromuscular blockade induced by snake venom Lys49 PLA₂ homologues (Fig. 7): the binding of the Lys49 PLA₂ homologues to hydrophobic domains in muscle plasma membrane promotes a non-enzymatic alteration of the membrane structure. As a consequence, there is a collapse of the ionic gradient and depolarization of both muscle fiber and nerve terminal, mainly due to re-equilibration of sodium and potassium ions concentration. The persistent cell depolarization could inactivate voltage-dependent sodium channels in the perijunctional zone. Consequently, the threshold of excitability of the muscle fiber rises out of the reach of the endplate potential; no action potential is triggered and the neuromuscular transmission is blocked. The depolarization of nerve terminal could increase the spontaneous release of acetylcholine, i.e. the frequency of miniature endplate potentials. The action potentials superimposed on the background level of nerve depolarization are reduced since the membrane potential is already shifted nearer to the sodium equilibrium potential. The reduced action potentials promote a decreased calcium influx and consequently a reduction of releasing of evoked acetylcholine. The muscle fiber membrane disruption induced by Lys49 PLA₂ homologues also promotes an increase in the concentration of cytosolic calcium that initiates a complex series of degenerative effects on muscle fiber. By this mechanism, *C. sinensis* extract efficiently did avoid the initial trigger.

Fig. 7. Molecular structure of snake venom Lys49 PLA₂ homologue (Gallacci & Cavalcante, 2010).

Basu et al. (2005) showed that the theaflavins-induced facilitation was dependent on the calcium concentration of the physiological solution pointing to an involvement of calcium in the facilitatory action of theaflavins. It is evident that the skeletal muscle can contract in the absence of external calcium, but under physiological conditions, when calcium is present in the medium, it induces the release of stored calcium from the sarcoplasmic reticulum in order to maintain the optimal integrity of the contractile mechanism (Endo, 1985). Considering that *C. sinensis* extract totally prevent the neuromuscular blockade induced by the myotoxin and calcium seems to be involved in the toxic mechanism of BthTX-I, by different proposed mechanisms as already discussed, the explanation of Basu et al. (2005) that *C. sinensis*, produces a facilitatory effect, via theaflavin, acting presynaptically as calcium modulating factor is also other possibility.

In spite of the hypothesis discussed here, the actual molecular mechanism involving the *C. sinensis* extract and the BthTX-I interaction remains to be cleared.

Here, when the efficacy of *C. sinensis* extract was assayed against the crude venom, 80% of the contractile response was found preserved even after two hours of the venom exposure (Fig. 2), a promising result, since venom has a complex composition, differently from BthTX-I. Histological analyses clearly showed the protective effect of *C. sinensis* extract against the myotoxic action of venom (Fig. 3E), showing the same positive correlation between neurotoxicity and myotoxicity induced by the venom and the myotoxin.

Whereas only the commercial theaflavin protected against the neuromuscular blockade of *Crotalus durissus terrificus* (de Jesus Reis Rosa et al., 2010), here, both theaflavin and epigallocatechin gallate, totally protected against the paralysis by *Bothrops jararacussu* venom (Fig. 4), a result better than that produced by *C. sinensis* extract, since the amount of these phytochemicals in the extract (as shown in Fig. 5) is lesser than that used in the neutralization assays. However, the *C. sinensis* extract contains a multitude of other compounds, which real participation against the toxic effects of venom must be assayed, hence using an *in vivo* model simulating the cronicallly black or green tea consumption (by humans) followed by subcutaneous injection of the venoms. A comparison between the treatment with commercial antivenom alone and commercial antivenom plus theaflavin or epigallocatechin gallate is also interesting.

It is well-known that *C. durissus terrificus* and *B. jararacussu* venoms act differently in inducing clinical symptoms as well as *in vitro* paralysis at skeletomotor apparatus. Considering that venoms were previously incubated with each commercial phytochemical, and that epigallocatechin gallate, the major catechin in green tea, totally inhibited the toxic compounds of *B. jararacussu* venom, but did not do so against the rattlesnake venom, it is reasonable to suggest that theaflavin inhibits both presynaptic and postsynaptic venom effects, whereas epigallocatechin gallate inhibits mainly postsynaptic venom effects of these snake venoms, a question that remains to be cleared.

5. Conclusion

Camellia sinensis leaves extract possesses inhibitory effect against the neuromuscular blockade induced by *Bothrops jararacussu* venom and also bothropstoxin-I, by an unclear mechanism of action. Altogether, the data suggest that theaflavin and epigallocatechin gallate have a strong participation on these protective effects.

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