RESEARCH ARTICLE

Heparin at low concentration acts as antivenom against *Bothrops jararacussu* venom and bothropstoxin-I neurotoxic and myotoxic actions

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ABSTRACT

Heparin has been shown to antagonize myotoxic effects of crotaline venoms. Here a very low heparin concentration (LHC) was examined in its ability to antagonize the neurotoxic/myotoxic effects of *Bothrops jararacussu* venom and its phospholipase A_2 myotoxin, bothropstoxin-I (BthTX-I), in an *in vitro* nerve-muscle preparation and in mice gastrocnemius. Normalization of results was done by assays with commercial antibothropic antivenom (CBA). LHC (1 IU/ml) added to the incubation bath reduced by 4- and 4.5-fold (*vs* 2.8- and 2.5-fold by CBA) the neuromuscular paralysis, by 5.4 and 4.4-fold (*vs* 2.5- and 13.3-fold by CBA) the percentage of fibers damaged and by 6- and 1.7-fold (*vs* 30- and 1.6-fold by CBA) the CK activity induced by *B. jararacussu* and BthTX-I, respectively. Protamine sulphate added 15 min after the incubation of the preparation with LHC+venom, avoided the LHC neutralizing effect against venom neurotoxicity. This strongly attests that given the polycationic nature of protamine, it probably complexed with the polyanionic heparin making it unattainable for binding to basic components of venom, reducing toxicity. Since heparin antagonism is generally stronger against venom effects than is myotoxin we discuss that other venom components than the BthTX-I are likely target for the antagonism promoted by the polyanionic heparin.

KEYWORDS: Antagonism, neuromuscular junction, neutralization, protamine

INTRODUCTION

Bothrops jararacussu snake venom has been studied since the early 1900s (Brazil, 1909) and its myotoxic action has been well documented. The notion that the venom presents also neurotoxic activity has come from frog nerve-muscle *in vitro* studies done over 25 years ago (Rodrigues-Simioni et al, 1983). Bothropstoxin-I (BthTX-I), a Lys49 PLA_2 (lysine in position 49) from *B. jararacussu* venom (Homsi-Brandeburo et al, 1988), considered its major toxin reproduces the myotoxic and neurotoxic effects of the venom (Rodrigues-Simioni et al, 1983; Homsi-Brandeburo et al, 1988), and both actions are probably partners in producing the severe effects of *B. jararacussu* envenoming (Milani-Junior et al, 1997; Ribeiro and Jorge, 1997).

Strategies for minimizing the severe tissue damage at the snakebite site, in which antivenom therapy has been unsuccessful, have been hastened in recent years (Kanashiro et al, 2002; Soares et al, 2004; Doin-Silva et al, 2008). Heparin is

a natural anticoagulant molecule whose acidic nature potentially allows its interaction with basic phospholipases A_2. *In vivo* study demonstrated that pre-incubation of heparin with two crotalid venoms (from *Agkistrodon contortrix laticinctus* and *Crotalus viridis viridis* snakes) or their myotoxins antagonizes significantly the myotoxicity of them (Melo and Ownby, 1999). Other studies have shown that heparin is able to antagonize *in vitro* the release of creatine kinase (CK) and lactodehydrogenase (LDH) from muscle incubated with *Bothrops jararacussu* snake venom or its major myotoxin, bothropstoxin-I (BthTX-I) (Melo and Suarez-Kurtz, 1988a; Melo et al, 1993) resulting in reduction of the myotoxic effects. Previously, Oshima-Franco et al (2001) reported that heparin at a concentration of 60 IU/ml was not able to significantly antagonize the myotoxic effect of BthTX-I, but produced a 100% protection against neuromuscular blockade. Whether the antagonizing action of heparin against myotoxicity reported in literature and interpreted as is due to acid-basic complexes formation is also unclear.

In this study, these two open questions were revisited. In this context, the concentration of heparin was drastically reduced to 1 IU/ml to avoid the facilitatory effect which had been observed with 60 IU/ml (Oshima-Franco et al, 2001). By using this approach, we investigated if the heparin anti-neurotoxic effect still persisted and/or if the anti-myotoxic effect reported by other authors became apparent; furthermore, we used protamine sulfate, which like heparin is an anticoagulant drug, but that differently is cationic in nature instead of anionic like heparin is. By using this approach we evaluated whether the benefits brought about by heparin were due to formation of acid-basic complexes (heparin + basic venom components) or not.

For comparative purposes the antibothropic natural effect of heparin was assessed in parallel with the one produced by the commercial antibothropic serum against the myotoxicity induced by *B. jararacussu* venom and BthTX-I in mouse phrenic nerve-diaphragm preparation (*in vitro* study) or after intramuscular injection in mouse gastrocnemius (*in vivo* study).

MATERIALS AND METHODS

Materials

B. jararacussu venom (Bjssu) was provided by Instituto Butantan (São Paulo, SP, Brazil); BthTX-I was purified from *B. jararacussu* venom as described by Homsi-Brandeburgo et al (1988), and modified by Cintra et al (1993). Briefly, crude venom was gel filtered on Sephadex G-75 and the catalytic active fraction SIII was re-chromatographed on SP-Sephadex C-25. BthTX-I (a Lys49-PLA_2 analogue, catalytically inactive, last eluting fraction) was dialyzed and lyophilized. Heparin - Liquemine® (25000 IU/ml – B1019) and Protamine 1000® (lot 701811 151) were purchased from Roche (Rio de Janeiro, RJ, Brazil); Commercial Bothropic Antivenom (CBA) (FUNED – lot 030611-12) was kindly donated by the Escritório Regional de Saúde (ERSA) from Piracicaba (SP, Brazil); Creatine kinase (CK) CK-NAC Bioclin kit (lot 099) was purchased from Quibasa Química Básica Ltd (Belo Horizonte, MG, Brazil).

Animals

Male Swiss white mice (25-30 gm) were supplied by the Animal Services Unit of the State University of Campinas (UNICAMP). The animals were housed at 25 ± 3° C on a 12 hr light/dark cycle and had access to food and water *ad libitum*. This work was approved by the University Committee for Ethics in Animal Experimentation (CEEA/Institute of Biology, UNICAMP, Protocol 792-1) and the experiments were done in accordance with the guidelines established by the Brazilian College for Animal Experimentation (COBEA).

Evaluation of neurotoxicity

Phrenic nerve-diaphragm preparations (Bülbring, 1946) (PND) were obtained from mice anesthetized with halotan (Cristália, Campinas, SP, Brazil) and killed by exsanguination. The diaphragm was removed and mounted under a tension of 5 gm in a 5 ml organ bath containing Tyrode solution (pH 7.4, 37°C) with the following composition (mM): NaCl 137; KCl 2.7; $CaCl_2$ 1.8; $MgCl_2$ 0.49; NaH_2PO_4 0.42; $NaHCO_3$ 11.9 and glucose 11.1, aerated with 95% (v/v) O_2 and 5% (v/v) CO_2. Supramaximal stimuli (4x thresholds, 0.1 Hz, 0.2 ms) delivered from a Grass S48 stimulator (Astro-Med Inc, W Warwick, RI, USA) were applied to the nerve through bipolar electrodes. Isometric muscle tension was recorded by a force displacement transducer (Load Cell BG-10 GM, Kulite Semiconductor Products Inc, NJ, USA) coupled to a physiograph (Gould, Model RS 3400) via a Gould universal amplifier (both from Gould Inc, Recording Systems Division, Cleveland, OH, USA). The preparations were allowed to stabilize for at least 20 min before addition of either 40 µg/ml Bjssu, 2.9 µM BthTX-I, LHC, 5 IU/ml, CAB, 120 µl/ml, or with a mixture of venom:LHC (40 µg/ml: 5 IU/ml) or BthTX-I:LHC (2.9 µM:5 IU/ml), and venom:CBA (40 µg/ml:120 µl/ml), BthTX-I:CBA (2.9 µM:120 µl/ml), and observed for 120 min. Preparations using the mixtures venom/toxin plus LHC/CBA were preincubated at 37°C for 30 min before the onset of experiments. Control experiments were done with Tyrode solution alone. Some protocols aiming to evaluate the venom- or toxin-heparin binding were carried out using protamine sulfate (0.8 IU/ml), a heparin-antagonist, 15 min after addition to bath of a preincubated (30 min at 37°C) venom:LHC (40 µg/ml:5 IU/ml) mixture.

Evaluation of myotoxicity

Histological and morphometric analysis

At the end of each experiment, the mouse phrenic nerve-diaphragm muscle preparation was rapidly removed from the bath and fixed in Bouin's fluid for 24-48 hr. After washing in distilled water, ethanol series dehydration and Historesin embedding (Leica Instruments Gmbh, Nubloch/Heidelberg, Germany) sections 2 µm thick were obtained (Leika RM 2035 microtome) and stained with 0.5% (w/v) toluidine blue (Vetec, SP, Brazil) in 5% (w/v) borax (Quimesp, SP, Brazil) for examination by light microscopy (LM). The extension of muscle damage was assessed by counting the number of fibers with alterations (edema, darkening, sarcolemmal disruption and myofibrils lysis) and the figures were expressed as percentage of the total number of cells counted in three non-overlapping, non-adjacent areas of each muscle (Oshima-Franco et al, 2001). The formula for calculating the % of damaged fibers was %F = [(%f x 100)/Tf) - 100, where %f is the number of fibers affected by venom or toxin and Tf is the total number of fibers (affected and non-affected)

in the section. This procedure was used in all experiments (controls and treated preparations, n = 3/each).

Measurement of CK release

Since CK release can not be measured in hemidiaphragm preparations, matched-*in vivo* groups of animals as for *in vitro* protocols of venom [(40 µg/ml), toxin (2.9 µM), venom:LHC (40 µg/ml:5 IU/ml), toxin:LHC (2.9 µM:5 IU/ml), venom:antivenom (40 µg/ml:120 µl/ml), and toxin:antivenom (2.9 µM:120 µl/ml)] were utilized to measure the plasma CK levels after injection in mouse gastrocnemius muscle. Two hours after injection, animals blood was collected from tail vein, stored in heparinized capillary tubes, centrifuged (3000xg, 3 min), and the plasma CK levels were determined using a diagnostic kit (CK-NAC Bioclin®). The CK activity was expressed as international units per liter (IU/L), where 1 unit is the amount of enzyme which catalyses the transformation of 1 µmol of creatine/min at 37°C.

Statistical analysis

Each experimental protocol was repeated from three to eight times and the results reported as the mean ± SEM were used for statistical comparison using ANOVA (Repeated Measures) followed by post-hoc Tukey test with a value of $P < 0.05$ indicating significance.

RESULTS

Heparin neutralized the neuromuscular blockade induced by Bjssu and BthTX-I

The graphic profiles of the preparations treated with Tyrode solution (control, n = 4), LHC (5 IU/ml, n = 6) and CBA (120 µl/ml, n = 6) are represented in Figure 1A. Only the preparation treated with CBA alone showed an initial and marked facilitatory response represented by increased twitch-tension in all experiments, which was significantly different from Tyrode control ($P < 0.05$). Figures 1B and 1C show the preparations treated with 40 µg/ml Bjssu or 2.9 µM BthTX-I, which presented 88 ± 2% (n = 6) and 95 ± 1.3% (n = 8), respectively, of neuromuscular blockade at the end of incubation (120 min). The neutralization assays (Figure 1B and 1C) showed that the neuromuscular paralysis induced by venom and toxin were significantly attenuated ($P < 0.05$) changing to 20 ± 6% (n = 6) and 21 ± 7% (n = 8), respectively, if incubated with LHC and to 32 ± 6% (n = 6) and 38 ± 6% (n = 6), respectively, if incubated with CBA at the end of the 120 min incubation.

Protamine abolished neutralization of neuromuscular blockade promoted by heparin

Figure 1D shows that 20 µl protamine (corresponding to 0.8 IU/ml) added 15 min after incubation of PND with Bjssu:LHC mixture prevented the antagonistic action of heparin against the neuromuscular blockade induced by Bjssu, which persisted even after washing the preparation. No significant difference was seen in the time elapsed to achieve neuromuscular paralysis caused Bjssu alone (88 ± 2%; n = 6) and when protamine was prior added to the bath containing the mixture Bjssu+heparin (94 ± 4%; n = 6)

Heparin neutralized the morphological changes produced by Bjssu and BthTX-I in the diaphragm

Figure 2 shows light micrographs of diaphragm incubated with Tyrode solution, *B. jararacussu* venom or BthTX-I and with pre-incubation either with heparin or commercial antibothropic serum. In all set of experiments fibers in different stages of necrosis as well normal fibers were present. Swollen darkened fibers predominated but fibers containing densely clumped myofibrils, or compacted masses of myofibrils intermingled with diffuse amorphous areas of sarcoplasm were present. The percentage of fibers with morphological alterations was: Tyrode control (8%), LHC alone (7%), CBA alone (7%), Bjssu (27%) and BthTX-I (40%), ($P < 0.05$). Pre-incubation of LHC or CBA with venom or BthTX-I significantly reduced the percentage of affected fibers in such a way that there was no statistic difference in relation to preparations incubated with Tyrode solution or with either LHC or CBA alone. Heparin reduced to a greater degree the percentage of fibers affected by venom than did

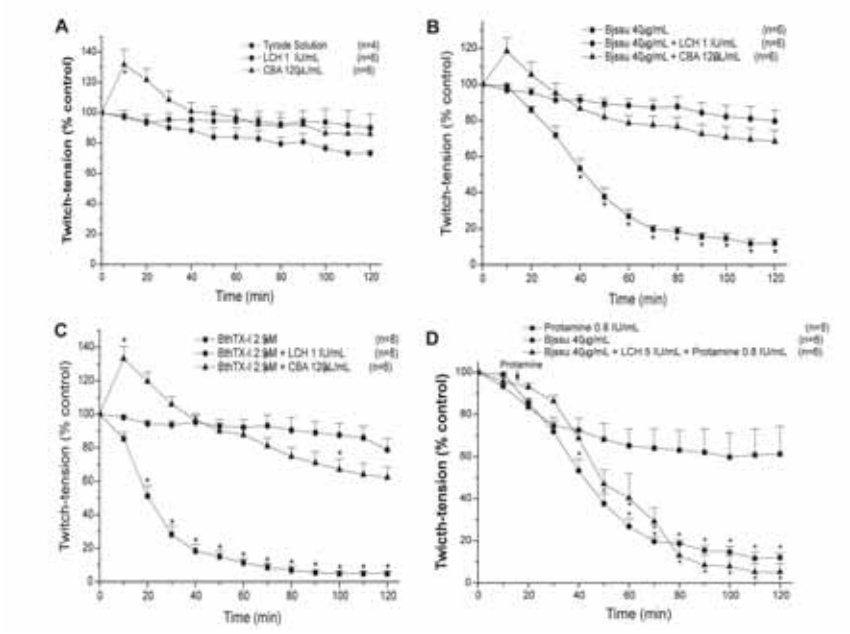

Figure 1. A. Controls of Tyrode solution, LHC and CBA on mouse phrenic nerve-diaphragm preparations, indirectly stimulated. **B** and **C.** Bjssu and BthTX-I neuromuscular action and its neutralization by LHC or CBA, respectively. **D**. Neuromuscular effects induced by protamine: Its neutralizing effect on venom:LHC mixture was likely due to complexation of protamine and heparin, therefore leaving Bjssu free for inducing its characteristic neuromuscular blockade. LHC, low heparin concentration; Bjssu, *Bothrops jararacussu* venom; BthTX-I, bothropstoxin-I. The points represent the mean ± SEM of the number of experiments indicated in parentheses. (*$P < 0.05$, when compared to control).

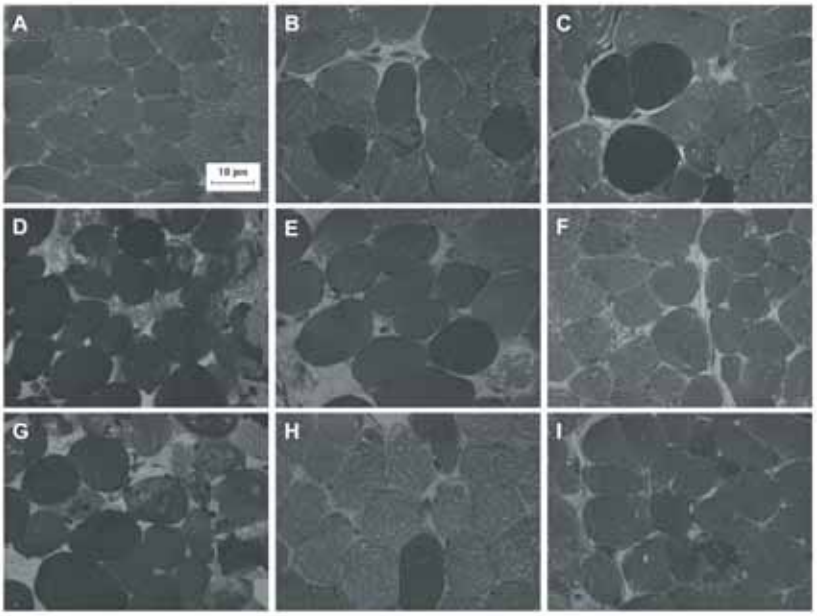

Figure 2. Light micrograph showing the histological appearance of diaphragm preparation after 120 min of indirect electrical stimulation and incubation in: (**A**) Tyrode Solution, (**B**) LHC 1 IU/ml, (**C**) CBA 120 µl/ml, (**D**) Bjssu 40 µg/ml, (**E**) Bjssu:LHC, (**F**) Bjssu:CBA, (**G**) BthTX-I 2.9 µM, (**H**) BthTX-I:LHC and (**I**) BthTX-I:CBA. Note the protection promoted by LHC and CBA against the myotoxicity induced by Bjssu and BthTX-I. LHC, low heparin concentration; CBA, commercial bothropic antivenom; Bjssu, *Bothrops jararacussu* venom; BthTX-I, bothropstoxin-I.

the commercial antiserum, as follows, 5% of fibers affected in Bjssu:LHC mixture and 11% in Bjssu:CBA mixture; a lesser efficiency had heparin in protecting against the pure myotoxin, *i.e.*, 9% of affected fibers incubated in BthTX-I: LHC mixture and 3% in BthTX-I:CBA mixture ($P > 0.05$).

Heparin neutralized the CK release from mouse gastrocnemius injected with Bjssu or BthTX-I

To co-validate the antagonizing effect of heparin and antiserum against venom-and pure myotoxin serum CK release was measured at 120 min after being injected in gastrocnemius. Figure 3 shows the serum CK activity (CK release). The basal CK activity (IU/l) was 104 ± 10 (n = 6) for saline control 119 ± 6 for LHC and 150 ± 13 for CBA, which were significantly different from the elevated 1454 ± 185 (n = 6) and 1531 ± 166 (n = 5) levels seen after Bjssu and BthTX-I injection, respectively. In contrast, the animals that received intramuscular (im) injection, either with venom:LHC or venom:CBA mixtures showed a significant shift of CK activity which dropped to 236 ± 40 (n = 6) and 47 ± 5 (n = 6, $P < 0.05$ compared to control), respectively. Interestingly, both the heparin and commercial antiserum were not as efficient in reducing the CK released by the Lys49 PLA_2 myotoxin (BthTX-I) as they were for the venom. Pre-incubation with LHC or CBA reduced to 900 ± 149 (n = 5) and 935 ± 135 (n = 5), respectively, the plasma CK levels in comparison to the observed with BthTX-I alone (1531 ± 166). However, these values were significantly different from those obtained with BthTX-I alone ($P < 0.05$).

DISCUSSION

Neurotoxicity

The present findings showed that when 1 IU/mL heparin is added to the venom- or bothropstoxin-containing bath the paralyzing effect was reduced by 4- and 4.5-fold, respectively. In comparison, when CBA is added to bath

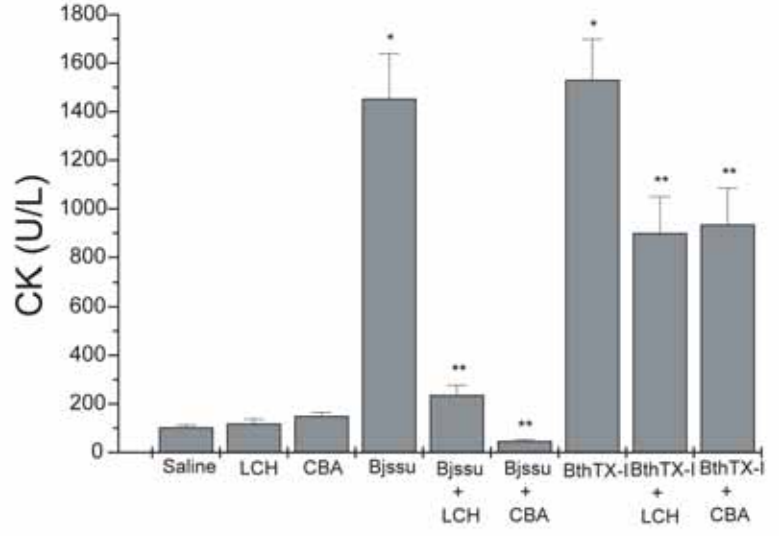

Figure 3. Measurement of plasmatic CK release, 120 min after saline, Bjssu, BthTX-I or Bjssu+LHC; BthTX-I+LHC; Bjssu+CBA, or BthTX-I+CBA injection in mouse gastrocnemius. CK, creatine kinase; LHC, low heparin concentration; CBA, commercial bothropic antivenom; Bjssu, *Bothrops jararacussu* venom; BthTX-I, bothropstoxin-I. (*$P < 0.05$ = significant difference in relation to saline control, **significant difference in relation to Bjssu and BthTX-I). The values represent the mean ± SEM of the number of experiments (n = 5).

the paralyzing effect is reduced in 2.8- and 2.5-fold, respectively. There was not neurotransmission facilitation with this heparin concentration, differently from the findings with 60 IU/ml (Oshima-Franco et al, 2001). In a different way, the CBA produced an initial 30% increase on the twitch tension in the first 10 min incubation which progressively returned to baseline by 40 min. This facilitatory effect in cholinergic neurotransmission produced by CBA had been already shown in isolated preparation (Oshima-Franco et al, 2000), alone or in venom:CBA or toxin:CBA mixtures, in agreement with the present findings. Recent study reported that the minimal concentration of heparin still able to produce facilitation on cholinergic neurotransmission (twitch tension increase) was 30 IU/ml, and that this concentration maintained the original ability to antagonize the neurotoxic effect of BthTX-I (Rodrigues et al, 2004). In the current study, a concentration 30-fold-lower (1 IU/ml) sustained such capacity of minimizing the neurotoxic effect, not causing any facilitation, as required for the purpose of the present study.

However, the heparin protection against bothropic venom effects seems to be reversible at least *in vitro*. In the current study, we showed that addition of protamine sulfate to the incubation bath containing Bjssu plus heparin completely inhibits the LHC antagonism. On the other hand, when protamine sulfate is incubated with Bjssu alone a total and irreversible blockade of muscle twitch tension is installed (data not shown). These findings give strong evidence that protamine forms a complex when in the bath with heparin. In contrast, when heparin is absent from bath protamine alone does not impede the neurotoxicity of Bjssu. Protamine, given its polycationic nature (Ando et al, 1973), is able to form stable inactive complexes with heparin, therefore it has been used as a specific antidote for neutralizing the heparin anticoagulant effect (Majerus et al, 1991).

Although accidents caused by *B. jararacussu* show no visible clinical signs of neurotoxicity, experimentally a number of *in vivo* or *in vitro* studies have shown that motor nerve fibers or nerve terminals can be affected by its venom. It abolishes contractions caused by direct and indirect electrical stimulation of skeletal muscle in mouse (Vital Brazil, 1966; Heluany et al, 1992; Oshima-Franco et al, 2000), chick (Heluany et al, 1992) and frog (Rodrigues-Simioni et al, 1983) neuromuscular preparations. These effects are reproduced by BthTX-I, a myotoxin which represents around 15% of total the venom (Homsi-Brandeburgo et al, 1988). A neurotoxic effect of Bjssu, also reproduced by a subfraction (13-15,000 Da) isolated from venom was detected in frog cutaneous pectoris nerve-muscle preparations, although the authors have concluded that the nerve terminal was not the prime site of action of the venom/subfraction (Rodrigues-Simioni et al, 1983). Agreeing with this, subsequent studies have given consistently focused on the preponderant myotoxic action of Bjssu and its major Lys-49 PLA_2 myotoxin (BthTX-I) (Melo and Suarez-Kurtz, 1988a; Melo and Suarez-Kurtz, 1988b; Oshima-Franco et al, 2000; Oshima-Franco et al, 2001; Randazzo-Moura et al, 2006). It was only when a concentration of BthTX-I, low enough that the neurotoxic effect cannot be concealed by the muscular one, that a pre-synaptic effect became evident (Oshima-Franco et al, 2004), then reassuring previous evidences on the existence of some pre-synaptic action of *B. jararacussu* venom (Rodrigues-Simioni et al, 1983).

This study does not give a clear explanation on how and why only low concentration of heparin (1 IU/ml) was able to antagonize the neuromuscular blocking effect evoked by Bjssu or BthTX-I. However, since heparin has a large therapeutic use in numerous disease conditions, such possibility should not be neglected. We can speculate that the highly-negative charge density of heparin contributes to attract positively-charged counterions of synaptic molecules, including that of AChE (through its collagen tail ColQ), thus promoting the neuro-protection. Either another possible explanation would be the heparin binding to components of venom and/or myotoxin, so neutralizing the expression of venom/toxin neurotoxicity. This latter possibility has been suggested to explain the heparin antagonism against myotoxicity of crotalid venoms, and was attributed to the anionic *vs.* cationic character of heparin *vs.* venom/toxin, respectively (Melo and Suarez-Kurtz, 1988b; Melo et al, 1993; Melo and Ownby, 1999).

Myotoxicity

In this regard, the mechanisms behind the anti-myotoxic effect of heparin have been stated as ought to: 1) the formation of acid-base complexes with basic myotoxins present in *Bothrops* venoms (Melo and Suarez-Kurtz, 1988a; Melo and Suarez-Kurtz, 1988b; Melo et al, 1993; Lomonte et al, 1994a; Lomonte et al, 1994b; Angulo et al, 2001) and/or to 2) the influence of heparin on PLA_2 present in many snake venoms (Lomonte et al, 1994b; Gutiérrez and Lomonte, 2003), affecting (Diccianini et al, 1990) or not (Condreas and Vries, 1964), the enzymatic activity of these proteins. In the present study, we demonstrated that differently from high concentration of heparin (60 IU/ml) (Oshima-Franco et al, 2001), a very low concentration (1 IU/ml) shows capacity to antagonize the myotoxicity caused by *B. jararacussu* venom and its main myotoxin in *in vitro* (morphometry of muscle damage) and *in vivo* (CK release) experiments. Thus, heparin is more effective in its antagonizing myotoxic effect against venom than against the myotoxin we can suggest that other venom components than the BthTX-I basic PLA_2 are likely target for the antagonism promoted by the polyanionic heparin.

In fact, besides phospholipases A_2, other venom components, such as metalloproteinases, serino proteases, among others (Gutiérrez and Lomonte, 1995; Gutiérrez and Rucavado, 2000) contribute to muscle damage, and could also be target for heparin neutralization. Damage is initiated as a consequence of loss of fiber sarcolemma integrity, followed by calcium influx and the forthcoming degenerative incomes comprising myofilament hypercontraction, mitochondrial alterations, activation of endogenous phospholipases and calcium-dependent proteases which amplify the muscle-damaging process (Gutiérrez and Lomonte, 2003). Concomitantly, it is launched an inflammatory response with release and activation of pro-inflammatory cytokines and other mediators, including nitric oxide, which could contribute to worsening the envenoming picture (Zamuner et al, 2005).

Heparin is a member of the glycosaminoglycan (GAG) family of carbohydrates, which includes the closely-related

molecule heparan sulfate. All of them participate in important biological processes. The interaction of GAGs with a bulk of cell and interstitial proteins, such as, growth factors, cytokines, cell adhesion molecules (Gandhi and Mancera, 2008), gives the dimension of their importance (Conrad, 1998). For instance, heparin is able to bind to extracellular matrix proteins, such as fibronectin or vitronectin (Lane and Adams, 1993; Lomonte et al, 1994b; Melo and Suarez-Kurtz, 1988b), leading to intracellular signalization triggered by ACh at the synaptic cleft and stability to tissue against toxic agents, among which could be candidates some components of snake venoms.

In summary, this work showed that heparin, when used a low concentration has greater efficiency than the commercial bothropic antivenom (CBA) to neutralize the neurotoxic activity of the *Bothrops jararacussu* venom and its major toxin. Low heparin concentration (LHC) paralleled in efficiency to CBA against the tissue damage caused by venom but was lesser efficient in relation to the toxin. Also, heparin paralleled to CBA in antagonizing the release of CK promoted by the toxin, but was less effective in antagonizing against the crude venom. The highest negative charge density of any known biological molecule (Cox and Lenninger, 2004) endows heparin binding to a high number of positively-charged molecules. Our study showed a novel finding, the principal myotoxin of *B. jararacussu* venom, the basic Lys-49 PLA_2 BthTX-I, is not the only target for heparin antagonism. Possible interactions of heparin with synaptic molecules and basic proteins of the extracellular matrix of the endomysium are discussed. Some of the differences seen in heparin effects whether used in low or high concentration are unclear.

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