Determination of Toxic Activities in Bothrops spp. Snake Venoms Using Animal-Free Approaches: Correlation Between In Vitro Versus In Vivo Assays

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ABSTRACT
The main purpose of this study is to investigate the in vitro toxic effects of 5 Bothrops spp. snake venoms, which are part of the antigenic mixture used for the production of Brazilian antivenom, and evaluate their correlation with the in vivo toxic activities of Bothrops spp. venoms. The correlation analysis could be helpful for the replacement of living animals experimentation for in vitro bioassay. Cytotoxicity, L-amino acid oxidase (LAAO), proteolitic (serine and metalloproteinase), hyaluronidase (Hyal), and phospholipase A2 (PLA2) activities were estimated and the correlation coefficient was determined for each activity in relation to lethality, edema, hemorrhage and necrosis induced in live animals by B. jararaca, B. alternatus, B. jararacussu, B. neuwiedi, and B. moojeni venoms. The lethal activity in mice was highly related to Hyal activity (r = 0.94, p < .05), edema related to PLA2 activity (r = 0.94, p < .05), whereas the necrotizing activity showed high correlation with LAAO activity (r = 0.83, p < .05). A very significant correlation between in vitro cytotoxicity and LAAO activities was also observed (r = 0.97, p < .05).

Key words: Bothrops venoms; in vitro assays; cytotoxic activity; lethal activity; hyaluronidase activity

Human envenoming by snakes of the genus Bothrops spp. is an important public health issue in the new world (Camey et al., 2002). This genus was responsible for 84% of the envenomation incidents, with mortality rates around 0.4% (Sistema de Informação de Agravos de Notificação, 2015). In Brazil, Bothrops species are responsible for 90% of all recorded snakebites (Jorge et al., 1997). Classic bothropic envenomation involves local and systemic effects. Edema, hemorrhage, and necrosis can lead to tissue loss and permanent disability. Systemic hemorrhage and intravascular coagulopathy can lead to acute kidney injury and cardiovascular shock, and are the main systemic complications caused by Bothrops spp. snakes (Albuquerque et al., 2013; White, 2005). The mentioned effects result from the integrated action of several venom components, such as metalloproteinases (known as Snake Venom MetalloProteinases—SVMPs), serine proteases (Snake Venom Serine Proteases—SVSPs), PLA2, L-amino acid oxidases (LAAO), hyaluronidases and others (Calvete, 2010). Although these families of molecules are well characterized as the main bothropic venom components, their relative abundance and specificity within different species venoms (or even individuals) are subjected to variations according to different parameters, such as age and geographic localization (Calvete, 2011).

In order to improve standardization of both the venoms and anti-venoms, the most widely accepted treatment for snake venomous bites, several biological activities of venoms are studied using both in vivo and in vitro methods (Theakston and Reid, 1983). However, it is generally agreed that in the current in vivo
assays a large number of animals are required for each toxic activity tested, causing considerable suffering (lethality, pain, hemorrhage, edema, necrosis, etc.). In addition, they are expensive and show little or no correlation with envenoming and therapy in humans (Theakston et al., 2003). From an ethical point of view, the increasing public concern on animal welfare has to be considered as a major driving force in the search for alternative methods to animal-based toxicity assays (Meier and Stocker, 1989; Purchase et al., 1998).

In this article, we present the determination of cytotoxicity, hyaluronidase (Hyal), LAAO, PLA2, and proteolytic activities of B. jararaca, B. alternatus, B. jararacussu, B. neuwiedii, and B. moojeni, the 5 Brazilian Bothrops spp. corresponding to the antigen mixture for producing the Brazilian anti-bothropic anti-venom. Thereafter, the correlation coefficient was determined for each activity in relation to lethality, edema, hemorrhage, and necrosis induced in living animals, previously determined by (Sanchez et al., 1992). The lethal activity in mice was highly related to Hyal activity, edema related to PLA2 activity, whereas the necrotizing activity showed high correlation with LAAO activity. Furthermore, very good correlation between in vitro cytotoxicity and LAAO activities was also observed.

MATERIALS AND METHODS

Venoms. Dehydrated venoms from Bothrops snakes, corresponding to the antigenic pool for producing the antivenom of bothropic venom (B. jararaca, B. alternatus, B. jararacussu, B. neuwiedii; and B. moojeni), were supplied by the Serpentarium of Fundação Ezequiel Dias, Belo Horizonte, Brazil. The venom of each snake was obtained by manual compression of the venom glands, pooled, centrifuged, lyophilized and stored at −20°C, following the recommendations of World Health Organization (1981). Solutions were prepared fresh by dissolving venoms in physiological saline (0.154 M NaCl). The protein content in crude venoms was determined by the procedure of Lowry et al. (1951) with bovine serum albumin as the standard.

Cytotoxic activities. MGSO-3 cell line derived from human primary breast cancer was used (Corrêa et al., 2013). These cells were characterized, immortalized, and kindly provided by Corrêa group. MGSO-3 cells were cultured in Dulbecco Modified Eagle’s Medium (DMEM, Sigma-Aldrich, St Louis, Missouri) supplemented with 10% fetal bovine serum (Thermo Scientific-Hyclone), 0.2% gentamicin (Gibco by Life Technologies) and kept in controlled atmosphere (10% CO2 incubator at 37°C). Cytotoxicity of the 5 venoms was tested against MGSO-3 by Alamar Blue assay according to (Damico et al., 2007), with modifications. In brief, 1 x 104 cells/well were plated in a 96-well microtiter plate and incubated for 24 h at 37°C. Cells were then exposed to 20 μg/ml of each venom in decreasing concentrations (dilution factor 1:2). After 24 h of incubation, 10% of Alamar Blue in DMEM was added to each well. The fluorescence was determined after 3 h at 540 nm of excitation and 590 nm of emission in a Synergy 2 (Biotek) fluorimeter. Values represent means of assays performed in quadruplicate. The effective concentration able to reduce by 50% (IC50) cell viability was determined from the dose-response curve, using the Graph Pad Prism 5 software.

Hyaluronidase activity. Hyaluronidase activity (Hyal) was measured as described by (Horta et al., 2014), with modifications, in a 96-well microtiter plate. The assay mixture contained 12.5 μg/ml of hyaluronic acid (HA) (Sigma-Aldrich), acetate buffer (0.2 M sodium acetate-acetic acid, 0.15 M NaCl) pH 6.0 and serial concentration (2.5–80 μg/ml) of Bothrops spp. venoms in a final volume of 100 μl. A standard curve with different concentrations of HA was used to estimate venom activity and the buffer was used as negative control. The assay mixture was incubated for 30 min at 37°C and the reactions were stopped by adding 200 μl of stop solution, containing 2.5% (w/v) cetyltrimethylammoniumbromide dissolved in 2% (w/v) NaOH. The absorbance was determined at 400 nm. Turbidity of the samples decreases proportionality to the enzymatic activity of hyaluronidase. The values were expressed as percentage of Hyal in relation to the negative and positive controls.

L-amino acid oxidases assay. To measure LAAO activity, an assay was conducted as described by (Bregge-Silva et al., 2012), with slight modifications, in a 96-well microtiter plate. The reaction mixture contained 100 mM Tris-HCl buffer, pH 8.5, L-leucine as substrate (5 mM), horseradish peroxidase (5 U/ml) and 2 mM OPD (as substrate for peroxidase). Each venom (2 μg) was incubated with the mixture at 37°C for 1 h. The reactions were stopped by the addition of 50 μl of 2 M H2SO4. The absorbance was determined at 490 nm by a Bio-Rad microplate reader model 680. The assay was performed in quadruplicate. One unit of LAAO activity was the amount of enzyme that produces 1 μmol of H2O2 per minute, under the specific conditions.

PLA2 activity. To access PLA2 activity, the EnzChek Phospholipase A2 Assay Kit (Life Technologies) was used, according to the manufacturer’s instructions. For each venom 2 μg were used. A solution of purified PLA2 10 U/ml in 1 x PLA2 reaction buffer was used as positive control and the same buffer without PLA2 was considered the negative control. Fluorescence was determined at 490 nm excitation and 570 nm emission.

Proteolytic activities. Proteolytic activity of venoms was tested in vitro upon different substrates coupled to a FRET system (Fluorescence Resonance Energy Transfer). Abz-LVEALYQ-EDDnp peptide comprises the amino acid sequence of the oxidized insulin B-chain which possesses a scissile peptide bond for SVMP at the position Ala14-Leu15. To measure the hydrolytic activity of serine proteases we used the substrate Abz-FLPRSRQ-EDDnp which contains the canonical Arg at the P1 position and is suitable to be cleaved by SVSP (Zelanski et al., 2015).

Assays were performed as described earlier (Schneider et al., 2014), in 96-well microplates using 1 μg of each venom and substrates at 47 mM (final concentration). Hydrolysis was monitored at 340 nm of excitation and 440 nm of emission, during 30 min at 37°C in Synergy2 (Biotek) equipment. To measure the serine protease activity, the 5 venoms were pre-incubated with 0.2 μM EDTA for 1 h, to prevent unspecified metalloproteinase degradation and then the same procedure was performed, using a biosensor containing the active site for serine proteases.

Determination of lethal, necrotizing, hemorrhage, and venom-induced edema activities. With the purpose of correlating in vivo versus in vitro toxic activities, the values of lethal, necrotizing, hemorrhage and venom-induced edema were retrieved from a work of Sanchez group previously reported (Sanchez et al., 1992).

Statistical analysis. Data were analyzed statistically by 1-way ANOVA or by 2-way ANOVA using the Graph Pad Prism 5 software. A p-value < .05 was considered significant.
RESULTS

Bothrops spp. snakes venoms of medical importance have cytotoxic activity (Oliveira et al., 2002). Using a mammary tumor cell line (MGSO-3) as an in vitro cell culture model, the cytotoxic potential of B. jararaca, B. alternatus, B. jararacussu, B. neuwiedi, and B. moojeni snake venoms was determined using 6 serial dilutions (0.31–20 μg/ml) of each Bothrops crude venom, as described in the previous section. As shown in Figure 1, treatment with all snake venoms resulted in a concentration-dependent inhibition of cell viability on MGSO-3 cultured cells. The level of cytotoxicity varied amongst species with the following rank order of potency: B. neuwiedi > B. jararacussu > B. moojeni > B. jararaca > B. alternatus. The relative cytotoxic activity of distinct venoms was determined as the amount necessary to kill 50% of cells or cytotoxic dose (CD<sub>50</sub>). B. neuwiedi B. jararacussu, and B. moojeni (4.07, 4.24, and 4.66 μg/ml, respectively) were the most cytotoxic, whereas B. jararaca (9.96 μg/ml) and B. alternatus (12.42 μg/ml) were the least cytotoxic venoms, respectively.

The Hyal was measured as described by (Horta et al., 2014), by incubating samples of the Bothrops spp. venoms (2.5–80 μg/ml) with HA, the substrate of the reaction. Figure 2 shows that all venoms exhibited significant Hyal activity in a concentration-dependent form. Venom from B. jararaca demonstrated increased activity compared with other Bothrops venoms tested. Venoms of B. neuwiedi and B. alternatus displayed intermediate activity followed by B. jararacussu and B. moojeni venoms, which presents low Hyal. The relative Hyal activity of the 5 distinct Bothrops spp. venoms was determined as the amount in μg/ml necessary to produce 50% of activity. B. jararaca (9.31 μg/ml) was the most active venom, B. neuwiedi and B. alternatus (14.7 and 14.38 μg/ml) were moderately active and B. jararacussu and B. moojeni (21.50 and 31.51 μg/ml) were the least toxic venoms, respectively.

LAAO activity was assessed according to the method described by Bregge-Silva et al. (2012). LAAO activity was observed in all tested venoms (Fig. 3A). The venoms of B.
neuwiedi, B. moojensi, and B. jararacussu showed higher LAAO activities (9.91, 9.24, and 9.16 U/mg/min, respectively). Venoms of B. jararaca and B. alternatus showed minor LAAO activities (3.92 and 3.28 U/mg/min, respectively). The level of LAAO activity varied amongst the species with the following rank order of potency: B. neuwiedi ≈ B. moojensi ≈ B. jararacussu > B. jararaca > B. alternatus.

The PLA2 activity of the venom samples was assessed using a fluorimetric method after incubating 2 μg of venom samples with phosphatidylcholine, the substrate of the reaction. Under these experimental conditions, PLA2 activity was detected in variable levels in all venoms tested (Fig. 3B). B. moojensi and B. jararacussu venoms had higher PLA2 activity than B. jararaca, B. neuwiedi, and B. alternatus venoms. The level of PLA2 activity varied amongst the species with the following rank order of potency: B. moojensi > B. jararacussu > B. jararaca ≥ B. neuwiedi > B. alternatus.

The proteolytic activities (metalloproteinase and serine proteases) of Bothrops spp. venoms were measured by FRET technique, using peptides whose sequence contains the active site for metalloproteinase and serine proteases, respectively, coupled with a fluorophore according to (Schneider et al., 2014). Under these conditions, and as shown in the Figure 3C, B. jararacussu and B. moojensi venoms exhibited higher serine protease activity than B. jararaca, B. alternatus, and B. neuwiedi venoms. Furthermore, the observed serine proteolytic activity of B. alternatus venom was lower than the other Bothrops venoms. In contrast, determination of metalloproteinase activity (Fig. 3D) showed that B. jararacussu venom had low proteolytic action and B. moojensi venom exhibited strong metalloproteinase activity, highest amongst all Bothrops venoms.

The comparison between 3 in vivo and 6 in vitro toxic activities presented by venoms from 5 different Bothrops spp., and the correlation of each activity with lethality, edema, and necrosis was the subject in this last part of this study (Tables 1 and 2). Cytotoxicity, LAAO, proteolitics, Hyal, and PLA2 activities were not equally distributed among the studied Bothrops spp. venoms (Table 1). The correlation coefficient was estimated for
each activity in relation to lethality, edema and necrosis induced by the venoms. Table 1 shows that lethal activity in mice was highly related to Hyal activity ($r = 0.94, p < .05$), and that edema was related to PLA$_2$ activity ($r = 0.94, p < .05$), whereas the necrotizing activity showed high correlation with LAAO activity ($r = 0.83, p < .05$). Very significant correlation between the cytotoxicity and LAAO in vitro activities was observed ($r = 0.97, p < .05$). Proteolytic activity of metalloproteases presented a low correlation with the in vivo toxic activities studied in this article. However, significant correlation between proteolytic activity by serine proteinases against in vivo venom-induced edema ($r = 0.68, p < .05$) and in vitro CD$_{50}$ ($r = 0.72, p < .05$) were observed.

**DISCUSSION**

The major goal of this work was to determine in vitro tests capable of replacing in vivo assays to study the toxic and lethal
TABLE 2. Correlation Coefficients (r) Between In Vivo and In Vitro Activities of Bothrops spp. Venoms

<table>
<thead>
<tr>
<th>Activities</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;* (µg/20 g mouse)</th>
<th>ED&lt;sub&gt;30&lt;/sub&gt;* (µg/mouse)</th>
<th>MHD* (µg/rat)</th>
<th>MND* (µg/rat)</th>
<th>CD&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyal (µg/50% act)</td>
<td>0.94*</td>
<td>0.25</td>
<td>0.07</td>
<td>0.14</td>
<td>0.31</td>
</tr>
<tr>
<td>PL&lt;sub&gt;A&lt;/sub&gt; (5% activity)</td>
<td>0.36</td>
<td>0.94*</td>
<td>0.07</td>
<td>0.44</td>
<td>0.64*</td>
</tr>
<tr>
<td>LAAO (U/mg/min.)</td>
<td>0.23</td>
<td>0.56</td>
<td>0.11</td>
<td>0.83*</td>
<td>0.97*</td>
</tr>
<tr>
<td>SVMV (RFU × 10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>0.41</td>
<td>0.11</td>
<td>0.05</td>
<td>0.11</td>
<td>0.02</td>
</tr>
<tr>
<td>SVSP (RFU × 10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>0.41</td>
<td>0.68*</td>
<td>—</td>
<td>0.36</td>
<td>0.72*</td>
</tr>
<tr>
<td>CD&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</td>
<td>0.20</td>
<td>0.71*</td>
<td>0.03</td>
<td>0.83*</td>
<td>—</td>
</tr>
</tbody>
</table>

*LD<sub>50</sub>, median lethal dose; ED<sub>30</sub>, edema dose; MHD, minimum hemorrhagic dose; and MND, minimum necrotizing dose as reported by Sanchez et al. (1992).

*p < .05

activities of crude snake venoms. For this purpose, toxic activities (CD<sub>50</sub>, LAAO, proteolytic, Hyal, and PL<sub>A</sub>) of 5 Bothrops spp. snake used in the production of bothropic anti-venom in Brazil (Furtado et al., 2010), were determined and correlated against lethality, edema, hemorrhage, and necrotic activities, previously demonstrated in rodents by Sanchez et al. (1992). We first optimized a cell-based assay to examine venom cytotoxicity. Cell-based assays have been used previously to investigate mechanisms of action and cytolytic applications for crude venom or purified toxins from snake venoms (de Melo Alves Paiva et al., 2011; Oliveira et al., 2002). The results obtained in this part of the work indicate that cytotoxicity of snake venoms can be determined by a simple in vitro assay using a readily available cell line. It can be useful to compare the toxicity of venoms from the genera Bothrops spp. Because no correlation exists between CD<sub>50</sub> and LD<sub>50</sub>, the application of CD<sub>50</sub> test to replace LD<sub>50</sub> test, is not possible for Bothrops venoms.

On the other hand, CD<sub>50</sub> activities present strong positive correlation with LAAO (r = 0.97, p < .05), necrosis (r = 0.83, p < .05), and edema (r = 0.71, p < .05). Snake venoms LAAOs (sv-LAAOs) are flavoenzymes which catalyze stereospecific oxidative deamination of a L-amino acid to give rise to alpha keto acids, ammonia and hydrogen peroxide (Fox, 2013). In some snake species sv-LAAOs constitutes up to 30% of the total venom protein content (Zeller, 1977). Experiments performed in cell culture show that exposure to sv-LAAO, triggered necrosis in a dose-dependent fashion. In vivo observations indicate that sv-LAAOs present in Bothrops spp. venoms are closely related to necrosis and represents an accidental form of cell demise resulting in early cell lysis, spillage of intracellular contents into the surrounding tissue, inflammation, and edema (Ande et al., 2006). The new point of our work is the demonstration of very significant correlation of both CD<sub>50</sub> and sv-LAAOs with necrosis (r = 0.83, p < .05). These results indicate that simple laboratory in vitro assays to measure LAAO activity can replace in vivo necrosis assays, at least in Bothrops spp. venoms.

There is general agreement that standard murine lethality assays (LD<sub>50</sub> of venom and ED<sub>30</sub> of anti-venom) lead to considerable suffering and death of animals, are expensive, hard to reproduce, and show no correlation with envenoming and therapy in humans. Indeed, in recent years the use of mouse lethality assay (LD<sub>50</sub> and ED<sub>30</sub>) and other in vivo tests have been questioned in Europe and the United States. Therefore, alternative assays must be developed to replace such tests (Theakston et al., 2003). One possible alternative in Bothrops venoms can be the measuring of Hyal activity, since the LD<sub>50</sub> and Hyal activities were highly related (r = 0.94, p < .05). Hyal are enzymes ubiquitously distributed in animal venoms from snake, bee, hornet, wasp, fish, spider, scorpion, caterpillar, and stingray (Fox, 2013). Hyal were recognized as producing a spreading response by hydrolyzing glycosaminoglycan and HA thereby allowing diffusion of fluids, facilitating the invasion of venom toxins into the victim’s organism and blood vessels, therefore acting as a catalyzer to the systemic envenomation (Ferrer et al., 2013; Horta et al., 2014). These last citations associated with the very high correlation between LD<sub>50</sub> and Hyal activities in Bothrops spp. venoms are the best support to our observation data related with the use of Hyal instead of mouse lethality assay.

The correlation between the levels of PL<sub>A</sub> and edematogenic effects (r = 0.94, p < .05) observed in this study is explained by the fact that PLs catalyze the hydrolysis of glycerophospholipids and promote the release of lysophospholipids and arachidonic acid, which are precursors of prostaglandins and leukotrienes, molecules involved in the inflammatory process characterized by increase by microvascular permeability formation, leukocyte recruitment into tissues and edema (Carvalho et al., 2013). Another example of edema formation through independent catalytic mechanisms by a PL<sub>A</sub> isolated from B. atrox venom (Kanashiro et al., 2002) is the degranulation of mastocytes, histamine liberation and formation of edema (Bonfim et al., 2009). The highly significant correlation observed between PL<sub>A</sub> activity and venom-induced edema, confirms our hypothesis that in vitro PL<sub>A</sub> activity can be used instead in vivo edema activity tests.

Surprisingly, no correlation was obtained when comparing SVMP activities and minimum hemorrhagic dose of the tested venoms. In the case of SVMPs, the targeting of these endopeptidases to their in vivo substrates is played by specific motifs/domains which would allow the co-localization of these enzymes and their respective molecular aims (Baldó et al., 2010; Escalante et al., 2006). It is known that SVMPs can cleave several components of basal membrane of endothelium and extracellular matrix, destabilizing capillary walls, which subjected to biophysical forces of blood flow, can be ruptured leading to blood leakage (Gutierrez et al., 2005). Other groups have been trying to establish an in vitro approach capable of replacing the in vivo measurement of hemorrhage (Bee et al., 2001; Rafael et al., 2008). However, the specificity of in vivo hydrolysis of certain high molecular weight substrates (eg, type IV collagen) by SVMPs appear to be more important than the simple turnover rate of these enzymes, measured here using Abz-LVEALQY-EDDnp.

SVSP activity was also addressed in this work. The hydrolysis of the specific substrate cleaved by these peptidases correlates positively with the edematogenic and cytotoxic effects measured previously (Sanchez et al., 1992). Serine proteases from snake venoms are enzymes acting mainly on components of the coagulation cascade resulting in haemostatic imbalance (Serrano, 2013). Besides, effects on the complement system and inflammation resulting in edema-inducing activities have already been reported (Menaldo et al., 2013; Zychar et al., 2010). Notwithstanding, there is scarce data about the effect of SVSP
on cell death/apoptosis. Further studies are required to clarify whether the correlation described in this work between SVSP activity and cytotoxicity might represent a real finding or not.

In conclusion, our study confirmed that in the Brazilian Bothrops spp. venoms the toxins are differently distributed in the distinct species. The knowledge of such differences can be of great relevance to the understanding of the effects of snake bite envenomation and antiserum production. The results obtained here indicate that a battery of methods can be used to determine the major toxic activities of Bothrops spp. snake venoms and the existence of high correlation between hyaluronidase and lethal activities shows that in vitro method can be an alternative method to animal-based toxicity assay.

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