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Comparative analysis of newborn and adult *Bothrops jararaca* snake venoms

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ABSTRACT

Different clinical manifestations have been reported to occur in patients bitten by newborn and adult *Bothrops jararaca* snakes. Herein, we studied the chemical composition and biological activities of *B. jararaca* venoms and their immunoneutralization by commercial antivenin at these ontogenetic stages. Important differences in protein profiles were noticed both in SDS-PAGE and two-dimensional electrophoresis. Newborn venom showed lower proteolytic activity on collagen and fibrinogen, diminished hemorrhagic activity in mouse skin and hind paws, and lower edematogenic, ADPase and 5′-nucleotidase activities. However, newborn snake venom showed higher L-amino oxidase, hyaluronidase, platelet aggregating, procoagulant and protein C activating activities. The adult venom is more lethal to mice than the newborn venom. *In vitro* and *in vivo* immunoneutralization tests showed that commercial *Bothrops* sp antivenin is less effective at neutralizing newborn venoms. These findings indicate remarkable differences in biological activities of *B. jararaca* venom over its development. We suggest that not only venom from adult specimens, but also from specimens at other ontogenetic stages should be included in the venom pool used for raising antibodies. Thus, *Bothrops* antivenin can efficaciously neutralize proteins lacking in the adult venom pool, especially those that promote more intense hemostatic disturbances in victims of newborn snakes.

1. Introduction

The jararaca (*Bothrops jararaca*)- is the main agent inflicting snake bites in São Paulo State, Brazil (Ribeiro and Jorge, 1997). This snake is found in southeastern South America, from Bahia State in Brazil to northern Argentina. In such vast area, they inhabit a number of habitats, such as Atlantic forest, semidecidous broadleaf forests, scrubs, cultivated fields, open areas and even in large cities (Sazima, 1992). The mean length of adult *B. jararaca* is 1 m long, but they may reach up to 1.6 m. Litter sizes vary from 5 to 22 neonates, showing a mean of 14. Births usually occur from February to March (Sazima, 1992; Campbell and Lamar, 2004), and the length of newborns is around 20 cm (Melgarejo, 2003). Feeding habits of *B. jararaca* shift during its growth: on the one hand, frogs are the main prey item (75%) for neonates, but birds, lizards, centipedes and small rodents have also been reported; on the other hand, adult snakes preferentially prey rodents (80%), but also lizards, frogs and birds (Sazima, 1991, 1992; Campbell and Lamar, 2004). Noteworthy, in newborn and juveniles of *B. jararaca* the tail tip is usually whitish or yellowish (Fig. 1), and as snakes change their diet to endotherms the tail tip becomes suffused with darker color, and identical to the remaining color pattern. In fact, neonates use the white

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Fig. 1. Specimen of newborn *Bothrops jararaca* snake, depicting an evident whitish tail tip.

tip tail to lure ectothermic preys (Sazima, 1991; Andrade et al., 1996).

Snakebites are a serious health problem, especially in Latin America, Africa and Asia (Warrell, 2010). Victims of bites by *B. jararaca* usually manifest local effects at the site of the bite (edema, ecchymoses, compartmental syndrome, blisters and necrosis) as well as systemic signs of envenomation, such as spontaneous bleeding (gingival bleeding, hematuria and epistaxis) and blood incoagulability (Santoro et al., 2008). Such clinical manifestations have been attributed to the activity of proteins and enzymes found in *B. jararaca* venom, as well as to pharmacological mediators engendered by them (Gutiérrez and Lomonte, 2003; Sano-Martins and Santoro, 2003).

Various toxins contribute to the development of the local inflammatory response evoked by *Bothrops* venoms: bradykinin-releasing enzymes (Vargaftig et al., 1974), lectins (Lomonte et al., 1990; Panunto et al., 2006), phospholipases A_2 (PLA_2) (Soares et al., 2001; Kanashiro et al., 2002; Ketelhut et al., 2003; Rodrigues et al., 2007), serine proteinases (Pérez et al., 2007), metalloproteinases (Gutiérrez et al., 1995; Gutiérrez and Rucavado, 2000; Rodrigues et al., 2001; Zychar et al., 2010) and L-amino oxidases (Stábeli et al., 2004, 2007; Izidoro et al., 2006). Hyaluronidases may also contribute indirectly to the exacerbation of the local reaction, by hydrolyzing hyaluronic acid present in the connective tissue, and potentiating thereby the diffusion and absorption of venom components to the blood stream (Girish and Kemparaju, 2006).

Hemostatic dysfunction evoked by *B. jararaca* venom is a complex phenomenon, eventually leading to consumption of blood coagulation factors and circulating platelets, platelet dysfunction, and secondary activation of fibrinolysis (Maruyama et al., 1990; Santoro et al., 1994, 2008; Santoro and Sano-Martins, 2004). Several *Bothrops* toxins take part on hemostatic dysfunction, e.g., thrombin-like enzymes, factor X and prothrombin activators, toxins that stimulate or inhibit platelet function, and hemorrhagins (Nahas et al., 1979; Maruyama et al., 1990, 1992; Kamiguti et al., 1991; Kamiguti et al., 1994; Santoro et al., 1994; Sano-Martins et al., 1997; Sano-Martins and Santoro, 2003; Santoro and Sano-Martins, 2004; Gutiérrez et al., 2005; Rucavado et al., 2005). In addition, nucleotidases present in *B. jararaca* venom, such as phosphodiesterases and 5′-nucleotidases (Sales and Santoro, 2008; Santoro et al., 2009) may disturb a plethora of physiological functions, including platelet function (Aird, 2002; Santoro et al., 2009) and contribute to local and systemic signs manifested by patients bitten by *B. jararaca*.

Several factors, such as seasonal variation, habitat, age, and sexual dimorphism are known to produce venom variation (Chippaux et al., 1991; Furtado et al., 2006; Menezes et al., 2006; Pimenta et al., 2007), although it is still controversial to which extent the diet influences the protein profile of snake venoms (Daltry et al., 1996; Sasa, 1999). In fact, intraspecies (geographical, seasonal and ontogenetic) variation in venom composition may account for the variability observed in severity and pattern of snakebite envenomation (for review see Warrell (1997)). Differences in the clinical picture and severity of people bitten by adult and young *B. jararaca* have been reported since the pioneer observations by Monteiro (1610), who noticed that patients bitten by "jararaca soatinga" (jararaca with white tail tip, Fig. 1) have intense bleeding and rarely survive. Later on, Casal (1817) reported that "(…) the jararaca, whose strain is the most abundant and fatal: the one with white tail tip is no more than one palm in length, and its venom does not have any known antidote: the being, whom it bit, soon manifests convulsions and bloody sweating, and expires in a short time". Such historic citations demonstrate that hemostatic disturbances are a conspicuous manifestation of the envenomation inflicted by young *B. jararaca*. At a much later time, Rosenfeld et al. (1959b) described that envenomation by young *B. jararaca* snakes did not elicit local signs of envenomation, but evoked blood incoagulability. In fact, a survey of clinical signs observed in patients bitten by *B. jararaca* revealed that a higher incidence of blood incoagulability and a lower frequency of necrosis, blisters and abscess were found in patients bitten by young snakes (Ribeiro and Jorge, 1989, 1990), which are in accordance with preliminary experimental evidence demonstrating discrepancies in coagulant and proteolytic activities of young and adult *B. jararaca* venoms (Rosenfeld et al., 1959a; Kamiguti, 1988; Furtado et al., 1991).

Ontogenetic variation in venom composition has been reported in a number of genera (Minton and Weinstein, 1986; Mackessy, 1988; Gutiérrez et al., 1990, 1991; Saravia et al., 2002), including *Bothrops* snakes (Gutiérrez et al., 1980; Kamiguti, 1988; Furtado et al., 1991; Chaves et al., 1992; López-Lozano et al., 2002; Saldarriaga et al., 2003; Guércio et al., 2006; Zelanis et al., 2007, 2010), but few studies showed in detail what are the changes occurring in the biological activities, chemical composition and immunoneutralization of *B. jararaca* venom during the passage from newborn to adult snakes. Herein, we addressed such question, taking into account a clinical point of view, by studying pooled venoms from newborn and adult *B. jararaca*. We show herein that ontogenetic variation occurs in venom during the development of *B. jararaca*, which are related to the clinical picture observed in patients bitten by either adult or young *B. jararaca* snakes, and that commercial *Bothrops* antivenin does not neutralize the newborn venom as efficiently as the adult one.

2. Material and methods

2.1. Reagents

Dithiothreitol (DTT), iodoacetamide, urea, thiourea, CHAPS, Na_2-EDTA, phenylmethanesulfonyl fluoride (PMSF), *o*-phenanthroline, azocasein, azocoll, *N*-benzoyl-D,L-arginine-*p*-nitroanilide hydrochloride (BAPNA), gelatin from porcine skin, bicinchoninic acid kit, L-leucine, *o*-phenylenediamine, horseradish peroxidase, bis (*p*-nitrophenyl) phosphate, ATP, ADP, AMP, cetyltrimethylammonium bromide, hyaluronic acid, bovine serum albumin, and peroxidase-conjugated anti-horse IgG (A9292) were purchased from Sigma (USA). Prothrombin, factor X and protein C purified from human plasma were purchased from Calbiochem (USA). The synthetic chromogenic substrates S-2765, S-2238, and S-2366 were purchased from Chromogenix (USA). Bovine, rat and rabbit fibrinogens were purified as described elsewhere (Santoro et al., 1999). DeStreak, Pharmalyte, and precast strips for isoelectric focusing were purchased from GE Healthcare (USA). Broad-range molecular mass markers were purchased from Bio-Rad (USA). All other reagents were of analytical grade or better.

2.2. Venom pools

Specimens of *B. jararaca* snakes born in captivity at the Laboratory of Herpetology, Institute Butantan, were used. Venom has been milked just after their birth, pooled, and lyophilized. In order to obtain the venom pool from adult snakes, male and female adult specimens of *B. jararaca*, maintained at the Laboratory of Herpetology, with a minimum 65 cm snout-vent length (Sazima, 1992; Janeiro-Cinquini, 2004) were milked. Following lyophilization, all venom samples were maintained at −20 °C until use.

2.3. Animals

All procedures involving the use of animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and were approved by the Ethical Committee for the Use of Animals of Institute Butantan (certificate 451/08). Male Swiss mice, weighing 20–25 g, and male Wistar rats, weighing 180–200 g, were supplied by the Animal House, Institute Butantan.

2.4. Content of proteins, neutral hexoses, lipids and inorganic constituents in snake venoms

Protein content in venom samples (0.5 mg/mL) was determined by BCA assay on microplates (Redinbaugh and Turley, 1986), using bovine serum albumin as standard. Neutral hexose content in venom samples (2 mg/mL) was determined by the phenol–sulfuric acid method (Dubois et al., 1956), using D-glucose as a standard. Instrumental neutron activation was applied for elemental venom analysis (Saiki et al., 1991). The content of total lipids (Zollner and Kirsh, 1962), phospholipids (Zilversmit and Davis, 1950) and cholesterol (Bioclin kit, Brazil) was also analyzed in venom samples (25 mg/mL).

2.5. Electrophoresis

Analysis by one- and two-dimensional gel electrophoresis was used to evaluate the protein profile of adult and newborn snake venoms.

2.5.1. One-dimensional gel electrophoresis

Venom samples (2 mg/mL) were homogeneized with equal volumes of sample buffer in the presence or absence of 2-mercaptoethanol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 9.0–18.5% gradient gels (Laemmli, 1970), and thereafter gels were silver stained (Blum et al., 1987) and analyzed with TotalLab TL100 software (USA).

2.5.2. Two-dimensional gel electrophoresis

Samples of adult and newborn *B. jararaca* venom pools (800 μg protein/mL) were dissolved in rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS, 0.002% bromophenol blue) containing 0.5% Pharmalyte (pH 3–10 linear) and 2.8 mg/mL DTT. Precast strips for isoelectric focusing (IEF) (13 cm, linear pH 3–10) were used for the separation of proteins in the first dimension; each strip was hydrated with 250 μL of each venom solution overnight at room temperature. The first dimension IEF was performed in an Ettan IPGphor 3 instrument (GE Healthcare, USA), using the following protocol: 300 V–540 V h; 500 V–500 V h (gradient); 1000 V–1000 V h (gradient); 8000 V–11300 V h (gradient); and 8000 V–16000 V h. Following IEF, strips were reduced and alkylated by sequential incubation with DTT (10 mg/mL) and iodoacetamide (25 mg/mL) in equilibration buffer (6 M urea, 75 mM Tris–HCl, 29.3% (v/v) glycerol, 2% SDS, 0.002% bromophenol blue, pH 8.8). To accomplish the second dimension, the previously reduced and alkylated strips were washed in SDS-PAGE running buffer and directly applied to 12% SDS-PAGE gels in a Protean II xi cell (Bio-Rad), which was connected to a Multitemp III cooling bath (GE Healthcare, USA) set at 10 °C. Gels were run at 1 W/gel until the dye front reached the gel bottom. Thereafter gels were silver stained (Blum et al., 1987) and scanned on a Epson Imagescanner III, with resolution of 300 dpi. In order to analyze statistically the variation in protein expression among gels, each sample of venom was run four times, and then images were analyzed with the software ImageMaster 2D 7.0 (GE Healthcare, U.S.A.).

2.6. Proteolytic activity

Proteolytic activity of venoms was estimated using four different substrates: azocasein, collagen, fibrinogen, and BAPNA.

2.6.1. Caseinolytic activity

Caseinolytic activity was determined as reported previously (Wang and Huang, 2002). Briefly, aliquots (85 μL) of 4.25 mg/mL azocasein were incubated for 90 min at 37 °C with 10 μL of two-fold serial dilutions of adult or newborn venoms, all dissolved in 50 mM Tris–HCl buffer, pH 8.0. The reaction was terminated by addition of 200 μL of 5% trichloroacetic acid at room temperature, and the samples

were centrifuged at 1000 *g* for 5 min. Supernatants (100 μL) were homogenized with 100 μL of 0.5 M NaOH, and the absorbance was measured at 450 nm in a SpectraMax 190 microplate reader (Molecular Devices, USA). One unit of enzymatic activity was defined as the amount of venom that causes an increase of 0.005 units of absorbance per min at 450 nm, and the specific activity was expressed as U/min/mg lyophilized venom.

2.6.2. Collagenolytic activity

Collagenolytic activity of venoms was determined using both a quantitative assay using azocoll as substrate (Vachova and Moravcova, 1993) and a zymographic assay (Barbaro et al., 2005). For the former assay, two-fold serial dilutions (200 μL) of adult and newborn venoms were incubated with 50 μL of 5 mg/mL azocoll solution, both in Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 3.0 mM NaH_2PO_4, 10 mM HEPES, 5.6 mM dextrose, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4), for 1 h at 37 °C, taking the care to homogenize samples every 5 min during incubation; the reaction was halted by placing samples on ice. After centrifugation for 3 min at 5000 *g*, the absorbance of the supernatant (175 μL) was measured at 540 nm, and the specific activity was calculated similarly as that of caseinolytic activity, except that one unit of enzymatic activity was defined as the amount of venom that causes an increase of 0.003 units of absorbance per min at 540 nm. Specific activity was expressed as U/min/mg lyophilized venom. For the latter assay, samples of venom pools (10 μg) were dissolved in non-reducing sample buffer and subjected to SDS-PAGE on 10% gels containing gelatin from porcine skin (2 mg/mL). Gels were washed with 2.5% Triton X-100 solution in the presence or absence of metalloproteinase inhibitors – 20 mM Na_2-EDTA and 3 mM *o*-phenanthroline – and incubated at 37 °C overnight in developing buffer (50 mM Tris, 5 mM $CaCl_2$, 0.02% NaN_3, pH 8.8), in the presence or absence of the same inhibitors. Gels were stained with Coomassie blue R-250 to visualize the regions of enzymatic activity, which correspond to the clear areas in gels.

2.6.3. Amidolytic activity

To assay the activity of serine proteases in venoms (Erlanger et al., 1961), samples (30 μL) of two-fold venom dilutions in Tris buffer (50 mM Tris–HCl, 20 mM $CaCl_2$, pH 8.0) were incubated with 140 μL of substrate solution (10 mM BAPNA, 50 mM Tris–HCl, 20 mM $CaCl_2$) for 1 h at 37 °C. The reaction was interrupted by the addition of 50 μL of 30% (v/v) acetic acid, and the absorbance was read at 405 nm in a SpectraMax 190 microplate reader. The coefficient of extinction of *p*-nitroaniline was considered as $\varepsilon^{1\ \mathrm{mM}}_{405\ \mathrm{nm}} = 9.65/\mathrm{cm}$, and specific activity was expressed as nmol of *p*-nitroaniline/min/mg lyophilized venom.

2.6.4. Fibrinogenolytic activity

Fibrinogenolytic activity was assayed as described elsewhere (Santoro and Sano-Martins, 1993). Briefly, aliquots (8 μL, 0.5 mg/mL) of venom samples were homogenized with 200 μL of bovine or rabbit fibrinogen solution (2 mg/mL clottable protein), both dissolved in Tyrode buffer, and incubated at 37 °C for different periods of time. Two samples of fibrinogen were also incubated with venoms in the presence of either 5 mM Na_2-EDTA or 8 mM PMSF (final concentrations), and the reaction was interrupted after 180 min of incubation. Fibrinogen hydrolysis was halted by addition of 200 μL of SDS-PAGE sample buffer, and samples were then subjected to SDS-PAGE (Laemmli, 1970). Gels were silver stained (Blum et al., 1987) and analyzed with TotalLab TL100 software (USA).

2.7. Edematogenic activity

Mice were injected into the subplantar area of the right hind paw with 1 μg of either adult or newborn venom dissolved in 50 μL of sterile 154 mM NaCl. As a control, the left hind paw received the same volume of vehicle. Paw edema was determined by measuring paw thickness with a digital caliper at 0.5, 1, 2, 4, 6 and 24 h after venom injection, and the results were calculated as the difference in thickness between right and left paws. Edema was expressed as the percentage increase in paw thickness (Nunes et al., 2007).

2.8. Nucleotidase activities

ATPase, ADPase and 5′-nucleotidase activities in venoms were assayed as described elsewhere (Sales and Santoro, 2008). Shortly, two-fold serial dilutions (50 μL) of venoms were incubated with 50 μL of 1 mM ATP, ADP or AMP, all in incubation buffer (0.1 M glycine–NaOH buffer, 3.8 mM $MgCl_2$, pH 8.9), in a humid chamber at 37 °C for 15 min, and the liberation of orthophosphate was colorimetrically determined. Absorbance was measured at 820 nm, and ATPase and ADPase specific activities were expressed in nmol of liberated orthophosphate/min/mg lyophilized venom, while 5′-nucleotidase specific activity was expressed in μmol of liberated orthophosphate/min/mg lyophilized venom. To assay phosphodiesterase activity (Sales and Santoro, 2008), samples of each venom pool (30 μL, 1 mg/mL) were incubated with 10 mM bis(*p*-nitrophenyl) phosphate (140 μL), both in incubation buffer, at 37 °C for 1 h. The reaction was interrupted by the addition of 50 μL of 50 mM NaOH, and the absorbance was read at 405 nm in a SpectraMax 190 microplate reader and corrected to a path length of 1 cm. The concentration of *p*-nitrophenol was determined using the coefficient of extinction $\varepsilon^{1\ \mathrm{mM}}_{405\ \mathrm{nm}} = 18.0/\mathrm{cm}$ (Sales and Santoro, 2008), and specific activity of phosphodiesterase was expressed in nmol of *p*-nitrophenol/min/mg lyophilized venom.

2.9. L-amino acid oxidase (LAAO) activity

LAAO activity was determined by a spectrophotometric assay using microplates (Kishimoto and Takahashi, 2001), using dilutions of H_2O_2 (64–0.5 nmol H_2O_2) in 50 mM borax–HCl buffer, pH 8.5, as a standard. Two-fold serial dilutions (10 μL) of *B. jararaca* venoms in 0.1 M glycine–NaOH buffer (pH 8.9) were incubated for 1 h at 37 °C with 90 μL of reactive mixture (5 mM L-leucine, 2 mM *o*-phenylenediamine, and 0.81 U/ml horseradish peroxidase in 50 mM borax–HCl buffer). The reaction was halted by adding 50 μL of 2 M H_2SO_4, and the absorbance was read at 492 nm in a SpectraMax 190 microplate reader. Specific

activity was expressed in nmol of H_2O_2/min/mg lyophilized venom.

2.10. Hyaluronidase activity

A previously reported method (Pukrittayakamee et al., 1988) was used to assay hyaluronidase activity. Two-fold serial dilutions (10 μL) of venoms in 0.2 M acetate buffer, pH 6.0, containing 0.15 M NaCl, were incubated with 40 μL of hyaluronic acid solution (5 mg/mL in the same buffer) at 37 °C for 15 min. The reaction was stopped by adding 150 μL of 2.5% cetyltrimethylammonium bromide in 2% NaOH, and the absorbance was read at 400 nm in a SpectraMax 190 microplate reader. Acetate buffer was used as a blank, and a sample containing only hyaluronic acid solution was used as a measure of 100% turbidity. Hyaluronidase activity of venoms were expressed as the amount of venom required to hydrolyzed 50% of the hyaluronic acid (i.e., 10 μg), and then expressed in degraded hyaluronic acid/min/mg lyophilized venom.

2.11. Phospholipase A_2 (PLA_2) activity

PLA_2 activity was carried out as described previously (Santoro et al., 1999). Briefly, venoms were two-fold serially diluted in 154 mM NaCl, and aliquots (2 μL) were added to 200 μL of reactive mixture (100 mM NaCl, 10 mM $CaCl_2$, 7 mM Triton X-100, 0.265% soybean lecithin, 98.8 μM phenol red, pH 7.6). The mixture was immediately homogenized, and the absorbance read at 558 nm for 5 min at 37 °C in a SpectraMax 190 microplate reader. A unit of PLA_2 activity was defined as the amount of venom that gives $\Delta A_{558\ nm} = 0.3$/min. The specific activity was then expressed as U/mg lyophilized venom.

2.12. Myotoxic activity

Myotoxic activity of *B. jararaca* venoms was determined as described elsewhere (Santoro et al., 1999). Swiss male mice were injected in the gastrocnemius muscle with either venom (1 mg/mL, 50 μL) or vehicle (154 mM NaCl). Blood was obtained at 3 and 24h after injection, and myotoxic activity was evaluated by the rise of serum creatine kinase (CK) levels in serum using a commercial kit (Bioclin, Brazil). Enzyme activity was expressed in U/L.

2.13. Coagulant activity

2.13.1. Minimum coagulant dose (MCD)

Minimum coagulant dose (MCD) (Theakston and Reid, 1983) was determined in samples of citrated plasma (MCD-P) and fibrinogen solution (MCD-F) from different animal species (bovine, rabbit, rat and human), as described elsewhere (Santoro and Sano-Martins, 1993; Santoro et al., 1999). MCD was defined as the least amount of venom that clotted a solution of plasma or fibrinogen in 60 s at 37 °C. Final venom concentrations ranging from 0.4 to 800 μg/mL were tested. All clotting times were measured on a fibrometer (BBL, Becton Dickison, U.S.A.) at 37 °C. The regression analyses of plots of venom concentration against clotting time were performed using the software CurveExpert version 1.40 (http://curveexpert.webhop.net/), which calculated the best fit and MCD values.

2.13.2. Prothrombin, factor X and protein C activators

Evaluation of direct activation of prothrombin, factor X and protein C by either venom was evaluated by a modification of a described protocol (Yamada et al., 1997). Aliquots (5 μL) of two-fold serial dilutions (1000–0.061 μg/mL) of each venom were added to 85 μL of 14 μM human prothrombin, 5 μM human factor X or 0.5 μM human protein C, all dissolved in incubation buffer (20 mM Tris, 140 mM NaCl, 1 mM $CaCl_2$, 0.1% BSA, pH 8.0). After 20 min of incubation at 37 °C, 100 μL of 10 mM of the synthetic substrates S-2765, S-2238 or S-2366 was added to the samples incubated with factor X, prothrombin or protein C, respectively. As a control of direct hydrolysis of synthetic substrates by venoms, synthetic substrates were incubated with venom samples in the absence of coagulation factors, under the same conditions, and analyzed likewise. The amount of thrombin, factor X*a* and protein C*a* generated by the adult or newborn *B. jararaca* venoms was determined by measuring the initial velocity of release of *p*-nitroaniline at 405 nm at 37 °C in a SpectraMax 190 microplate reader, at the kinetics mode. Specific activities were expressed as μmol *p*-nitroaniline/min/mg lyophilized venom.

2.14. Activity on platelet aggregation

Blood platelets were washed from human blood as described elsewhere (Santoro et al., 1999), except that aliquots of platelet rich plasma were distributed in 1.5-mL centrifuge tubes and centrifuged at 10,000 *g* for 30 s. Platelet pellets were resuspended in calcium-free Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 3 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM HEPES, 0.35% bovine albumin, 5.6 mM dextrose, 0.02% apyrase, 50 ng/mL PGE_1, pH 6.2), and this procedure was repeated once more. Finally, platelet pellets were resuspended in Tyrode buffer containing calcium (137 mM NaCl, 2.7 mM KCl, 3 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM HEPES, 0.35% bovine albumin, 5.6 mM dextrose, 2 mM $CaCl_2$, pH 7.4), and platelet counts were determined in a Serono-Baker hematological counter, model 9020 + AX, and adjusted to 300×10^9/L. Platelet aggregation was monitored in a platelet aggregometer, model 560VS (Chrono-log, USA), with bar stirring speed set at 1000 rpm and constant temperature maintained at 37 °C. To evaluate the effect of snake venoms, 10 μL of two-fold serial dilutions of venoms (1000–62.5 μg/mL) in Tyrode solution containing calcium were added to 400 μL of a pre-warmed suspension of washed platelets. Five minutes after the addition of venom, the percentage of platelet aggregation was recorded and used to calculate the median effective dose (ED_{50}). To determine the ED_{50} for each venom, 5 μg/mL collagen (Chrono-log, EUA) (final concentration) was used as a reference of 100% platelet aggregation.

2.15. Hemorrhagic activity

Male Swiss mice were injected with aliquots of 50 μL of sterile 154 mM NaCl containing 16–0.5 μg venom of *B. jararaca* venoms into the shaved abdominal skin (5 mice/

dose). After 2 h, animals were euthanized in CO_2 chamber, the abdominal skin was removed, and the hemorrhagic halos were cut, fragmented and added to tubes containing 4 mL of Drabkin reagent. The material was incubated at 37 °C for 24 h in the dark, and thereafter tubes were centrifuged at 5000 *g* for 5 min. The absorbance of the supernatant was read at 540 nm, and then hemoglobin concentration was calculated using the formula described elsewhere (Gonçalves and Mariano, 2000). MHD was defined as the minimal concentration of venom able to induce an increase of three times in hemoglobin concentration in relation to that of a control tissue injected only with vehicle (Peichoto et al., 2007).

The intensity of local hemorrhage was also determined in hind paws used for evaluating the edematogenic activity of venoms (Gonçalves and Mariano, 2000). Briefly, groups of mice ($n = 6$) were injected i.pl. in the right and left hind paws with venom and vehicle, respectively, and 2 or 24 h later they were euthanized in a CO_2 chamber. Hind paws were removed at the level of tibiotarsal joint, weighed, fragmented and added to tubes containing 3 mL of Drabkin reagent. Following incubation in the dark at 37 °C for 24 h, tubes were centrifuged at 13,000 *g* for 5 min, and the supernatants were read at 540 nm. Hemorrhage – i.e., the difference between the hemoglobin content of paws injected with venom and those injected with vehicle – was expressed as mg hemoglobin/g of tissue.

2.16. Antibody titers for adult and newborn B. jararaca venom in Bothrops antivenin

Microplates (Nunc, USA) were coated with 100 μL/well of adult or newborn *B. jararaca* venom (10 μg/mL) dissolved in 0.1 M carbonate buffer, pH 9.6. The plates were incubated at 4 °C for 18 h in a humid chamber, washed 3 times with washing buffer (PBS pH 7.4 containing 0.05% Tween 20), and blocked with 3% bovine serum albumin in carbonate buffer (200 μL/well) for 2 h at 37 °C in a humid chamber. Subsequently, commercial *Bothrops* sp antivenin (Institute Butantan, lot 0611203) was two-fold serially diluted in incubation buffer (PBS pH 7.4 containing 1% BSA, 0.05% Tween 20), and 100 μL thereof was pipetted per well. After 1 h incubation at 37 °C in a humid chamber, microplates were washed and incubated with 1:10,000 peroxidase-conjugated anti-horse IgG (100 μL/well). Thereafter, microplates were washed, and the color reaction was developed by pipetting 100 μL/well of the substrate solution (1 mg/ml *o*-phenylenediamine and 0.03% H_2O_2 in citrate buffer, pH 5.0). The reaction was halted by adding 50 μL/well of 30% (v/v) H_2SO_4. Antibody titers against each venom pool were determined as the reciprocal of the highest dilution that gives an absorbance higher than the blank plus 0.05 at 492 nm.

2.17. Western blotting

Gels from two-dimensional electrophoresis (see above) were used for transference in Western blotting. Proteins were transferred to 0.2-μm nitrocellulose membranes in a semi-dry system (Bio-Rad) at 15 V for 2 h. Membranes were then blocked, and incubated with 1:1000 commercial *Bothrops* antivenin (Institute Butantan, 0611203) and subsequently with 1:8000 peroxidase-conjugated anti-horse IgG. The reaction was developed with diaminobenzidine as previously described (Pukac et al., 1997; Santoro et al., 2004).

2.18. Lethal dose 50% (LD_{50}) and its neutralization by antivenin

Lethal toxicity of either venom was determined in male Swiss mice. Each venom pool was two-fold serially diluted in 154 mM NaCl (0.285–18.240 mg venom/kg body weight), and 0.5 mL of each venom dose was injected i.p. into mice (6 mice/venom dose). The number of deaths occurring within 48 h was used to calculate LD_{50} (Litchfield and Wilcoxon, 1949). In order to determine the ability of commercial *Bothrops* sp antivenin (Institute Butantan, lot 0611203; each mL neutralizes 5 mg of *Bothrops* venom) to neutralize venom, 3 LD_{50} of adult (4.86 mg/kg) or newborn (11.28 mg/kg) *B. jararaca* venom were incubated with different volumes of antivenin (300–25 μL/mg venom) for 1 h at 37 °C, and then centrifuged at 2500 *g* for 10 min. The supernatant was injected i.p. into mice, and after 48 h the number of survivors was determined. The neutralizing capacity of antivenin was expressed as effective dose 50% (ED_{50}), defined as μL of antivenin/mg of venom at which half of the injected animals survived after 48 h (Gutiérrez et al., 1990). The values of LD_{50} and ED_{50}, as well as the statistical comparison between these values, were calculated as described elsewhere (Litchfield and Wilcoxon, 1949), using an algorithm specifically created for Stata™ 8.0.

2.19. Statistical analyses

Two-way analysis of variance (ANOVA) with repeated measures, followed by Tukey's test, was used to compare results from edematogenic activity. Myotoxic activity was compared by one-way ANOVA, followed by Tukey's test. Hemorrhage intensity in hind paws was compared by Student's t test. Statistical analyses were performed using the software STATA™, version 8.0, and SigmaStat, version 3.5. Differences with $p < 0.05$ were considered statistically significant. Data were expressed as mean ± SEM.

3. Results

3.1. Chemical properties

The levels of proteins and hexoses were similar in the venom pools from adult and newborn snakes (Table 1). On the other hand, the levels of total lipids was meager in both venoms, but the venom pool from adult snakes showed a concentration about three times higher than that of newborns. The levels of phospholipids and cholesterol were lower than the detection limit of the methods used for determination of these compounds. The levels of inorganic constituents were quite similar in both venom pools, except for strikingly high levels of K and Rb in the newborn *B. jararaca* venom (Table 1).

Table 1
Content of proteins, neutral hexoses, total lipids and inorganic constituents in newborn and adult *Bothrops jararaca* pooled venoms.

	B. jararaca venom	
	Adult	Newborn
Proteins (μg protein/mg venom)	594.82	612.29
Neutral hexoses (μg hexose/mg venom)	28.38	31.25
Total lipids (μg lipid/mg venom)	7.15	2.54
Inorganic constituents[a]		
Na (mg/g venom)	13.68 ± 0.12	12.59 ± 0.16
K (mg/g venom)	1.3 ± 0.23	26.82 ± 0.47
Cl (mg/g venom)	1.02 ± 0.11	1.62 ± 0.07
Ca (mg/g venom)	0.842 ± 0.041	1.694 ± 0.069
Zn (mg/g venom)	0.919 ± 0.004	0.699 ± 0.003
Rb (μg/g venom)	7.46 ± 0.09	131.4 ± 0.80
Br (μg/g venom)	7.854 ± 0.099	4.836 ± 0.061
Se (μg/g venom)	4.062 ± 0.035	1.817 ± 0.030
Cs (ng/g venom)	14.2 ± 4.5	64.6 ± 6.2

[a] Results of a single determination. The uncertainty of concentration was calculated using statistical errors in the measurements of counting rates of samples and standards.

3.2. Electrophoretic profiles

One-dimensional electrophoretic profiles showed many band similarities between both venom pools, under reducing and non-reducing conditions (Fig. 2). However, protein bands on the range 55.0–85.0 kDa were preferentially expressed in *B. jararaca* newborn venom, under reducing and non-reducing conditions, demonstrating that they are single-chain proteins. On the other hand, more intensely-stained bands were observed in the ranges of 14.7–16.0 kDa and 21.0–23 kDa, under reducing conditions,

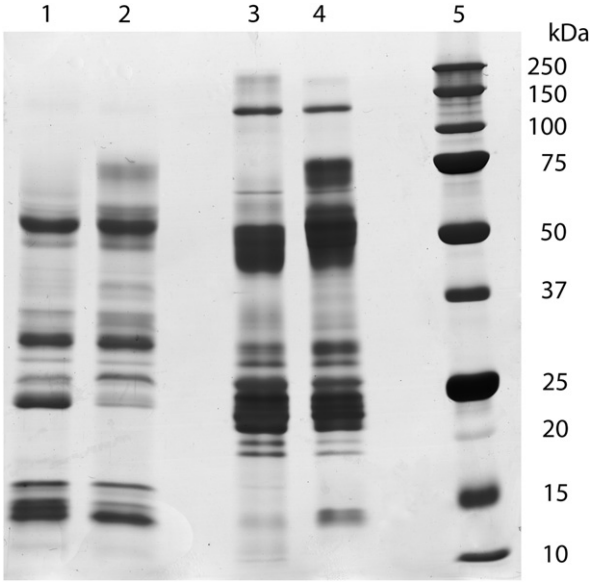

Fig. 2. Electrophoretic profiles of adult (lanes 1 and 3) and newborn (lanes 2 and 4) *B. jararaca* pooled venoms in SDS-PAGE (9.0–18.5%), under reducing (lanes 1 and 2) and non-reducing (lanes 3 and 4) conditions. Each lane was loaded with 10 μg of venom. Proteins were silver stained.

in the adult venom (Fig. 2). The analysis of two-dimensional electrophoresis (Fig. 3) enlarged the scope of the one-dimensional analysis. By computational analyses of the images, two hundred and fifty spots were revealed in the pooled venoms, so that 154 (61.6%) were present in both venoms, and 96 (38.4%) were preferentially expressed in either the newborn or the adult venom. Proteins in the range of 55.0–85.0 kDa, which were more densely stained in one-dimensional gels, were also observed in the form of several acidic spots (pI 3.5–7.0) in two-dimensional gels from the newborn venom (green box, overlay, Fig. 3). The adult venom is largely dominated in this region by a single spot with pI of 4.6 and molecular mass of approximately 51.0 kDa (Fig. 3, adult). On the other hand, basic protein spots were preferentially expressed in the adult venom (yellow box, overlay, Fig. 3). In addition, spots with molecular masses of 21.0–23.0 kDa (pI of 5.5–7.0) were much more pronounced in the adult venom (orange box, overlay, Fig. 3).

3.3. Proteolytic activity

The adult *B. jararaca* venom showed a collagenolytic activity about seven times greater than that from newborn snakes (Table 2). Similarly, when venom pools were submitted to electrophoresis in gels containing denaturated collagen (gelatin) (Fig. 4), marked gelatinolytic activity was noticed in the adult venom, especially around 58.0 kDa (Fig. 4a). In the region below 50.0 kDa, the profile of gelatinolytic activity was similar between both venoms, whereas discrete areas of lysis were exclusively observed in the newborn venom in the high molecular mass region (204.0–126.0 kDa) (Fig. 4a). In both venoms, gelatinolytic activity was totally or markedly reduced by incubation with metalloproteinase inhibitors (Fig. 4b). Unlike collagen, which is a natural substrate for snake venom proteinases, casein may be considered a foreign substrate for those enzymes. Thus, when the proteolytic activity of both venoms was tested on azocasein, similar activities were noticed, but such activity was markedly lower than that elicited by azocoll (collagen) (Table 2). The lower proteolytic activity of *B. jararaca* venom on casein compared to that on collagen demonstrates thereby the enhancement in the structure-function of venom proteases over evolution. To study the activity of serine proteinases, the synthetic substrate BAPNA was used; no remarkable difference was observed in the intensity of hydrolysis of BAPNA between both venoms (Table 2).

Venoms from adult and newborn *B. jararaca* hydrolyzed the Aα chain of both rabbit and bovine fibrinogen, but the newborn venom took longer to degrade fibrinogen (Fig. 5). In fact, the adult venom even partially hydrolyzed the Bβ chain of bovine fibrinogen, but this process was noticeably slower than the hydrolysis of Aα chain; no degradation of Bβ chain was elicited by the newborn venom. Addition of PMSF to venom samples (lanes 9) could not completely inhibit Aα chain hydrolysis of rabbit and bovine fibrinogen, but venom samples incubated with EDTA (lanes 8) showed a marked inhibition of Aα chain degradation. This finding evidences that metalloproteinases are the main enzymes involved in fibrinogenolysis in both adult and newborn *B. jararaca* venom.

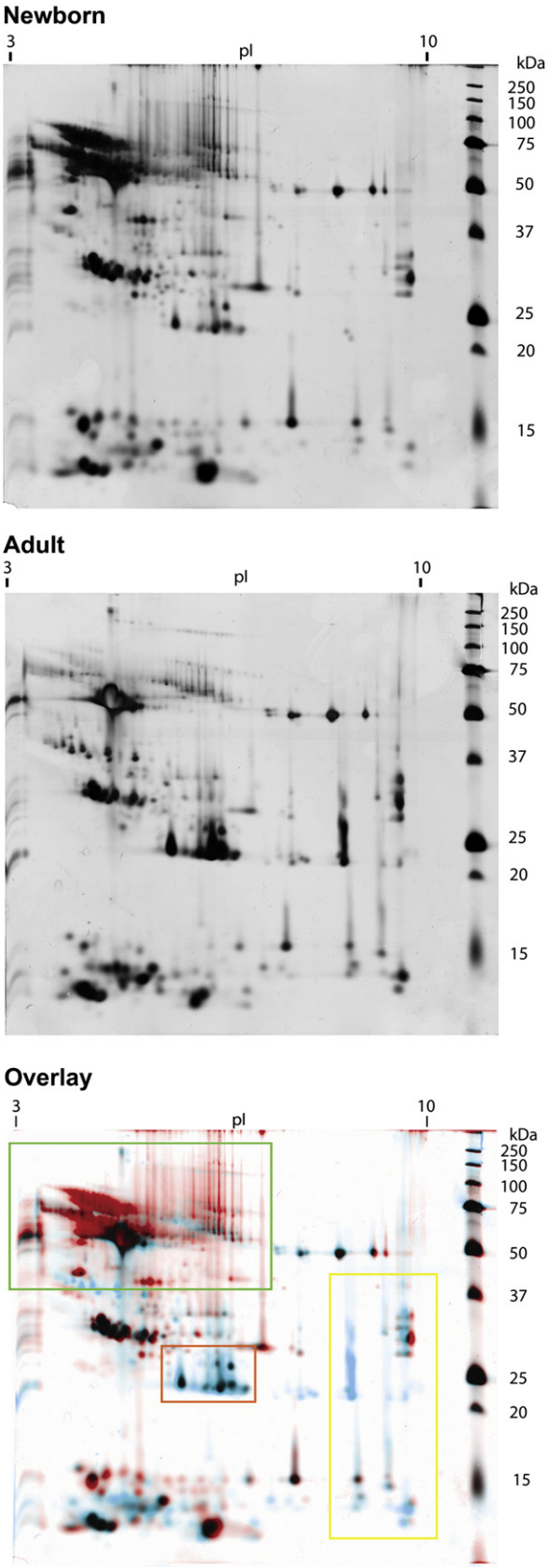

Table 2
Enzymatic activities of adult and newborn *B. jararaca* pooled venoms.

	B. jararaca venom	
	Adult	Newborn
Proteolytic activity on azocoll (U/min/mg venom)	2.99	0.49
Proteolytic activity on azocasein (U/min/mg venom)	0.54	0.50
Proteolytic activity on BAPNA (nmol p-nitroaniline/min/mg venom)	32.91	27.24
5′-nucleotidase activity (μmol orthophosphate/min/mg venom)	3.11	2.03
ADPase activity (nmol orthophosphate/min/mg venom)	924.02	588.35
ATPase activity (nmol orthophosphate/min/mg venom)	364.07	301.82
Phosphodiesterase activity (nmol p-nitrophenol/min/mg venom)	2.16	2.08
L-amino oxidase activity (nmol H_2O_2/min/mg venom)	26.04	34.87
Hyaluronidase activity (μg of hydrolyzed hyaluronic acid/min/mg venom)	20.65	35.65
Phospholipase A_2 activity (U/mg venom)	73.15	121.93

3.4. Edematogenic activity

Edematogenic activity of the adult venom was greater than that induced by the newborn venom, and statistically significant differences between groups were observed at 1, 6 and 24 h (Fig. 6).

3.5. Nucleotidase, PLA_2, L-amino oxidase and hyaluronidase activities

5′-nucleotidase and ADPase activities were ca. 50% higher in the adult venom. However, ATPase and phosphodiesterase activities were similar between both venoms (Table 2). L-amino oxidase, hyaluronidase and PLA_2 activities were higher (30–50%) in the newborn venom (Table 2).

3.6. Myotoxic activity

The elevation of serum CK levels elicited by the adult (841.8 ± 64.6 U/L, $n = 6$) and the newborn (947.2 ± 142.4 U/L, $n = 6$) venoms was remarkably higher than that elicited by saline (223.4 ± 61.8 U/L, $n = 6$) at 3 h, but no statistically significant difference was noticed between venoms. At 24 h after venom injection, serum CK levels had returned to normal (data not shown).

Fig. 3. Comparison of two-dimensional gel electrophoresis profiles of newborn and adult *B. jararaca* pooled venoms. Gels were run under identical conditions and silver stained. To provide a comparison of similarities between protein spots in venoms, colorized images from the newborn (red) and adult (cyan) venom gels were overlapped in Photoshop CS3 (Overlay); black spots reveal overlapping spots present in both venoms. The green box shows protein spots preferentially expressed in the newborn venom, while the yellow and orange boxes show spots preferentially expressed in the adult venom (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

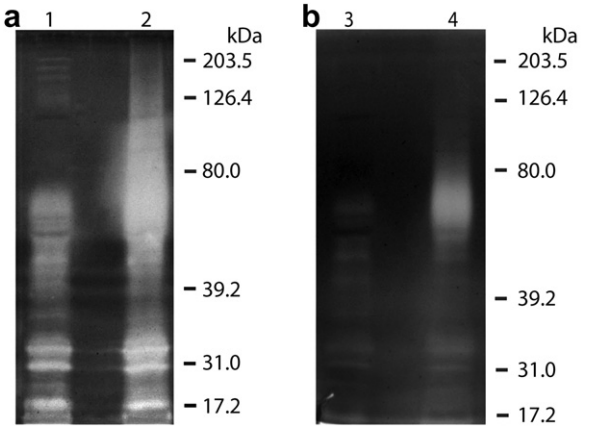

Fig. 4. Zymography for evaluating the gelatinolytic activity of newborn (1, 3) and adult (2, 4) *B. jararaca* venom. Following electrophoresis, gels were incubated in the absence (a) or presence (b) of 20 mM Na_2-EDTA and 3 mM *o*-phenanthroline.

3.7. Hemorrhagic activity

The adult venom showed a higher hemorrhagic activity than that of the newborn venom, since its MHD was about 40% lower than that of newborn snakes (Table 3). Accordingly, hemorrhage in mouse paws injected with the adult venom was greater than that observed in mice injected with the newborn venom at 24 h ($p = 0.028$), and apparently at 2 h ($p = 0.388$) (Table 3).

3.8. Coagulant activity

Coagulant activity of the newborn venom was remarkably higher than that of the adult venom, and such difference achieved values about 29, 9 and 3 times lower in rabbit, human and rat plasmas, respectively (Table 4). Adult and newborn venoms were more active on rabbit plasma, followed by human and rat plasmas. In regard to the thrombin-like activity, both venoms were not able to coagulate rabbit and rat fibrinogen, but they did coagulate bovine fibrinogen similarly (Table 4). In regard to vitamin K-dependent coagulation factors, the newborn venom was noticed to be ca. 36, 31 and 43 times more potent to activate prothrombin, factor X and protein C, respectively, than the adult venom (Table 4). Among those activities, prothrombin activation was the most exuberant, followed by activation of factor X. Protein C was the least activated by *B. jararaca* venom (Table 4).

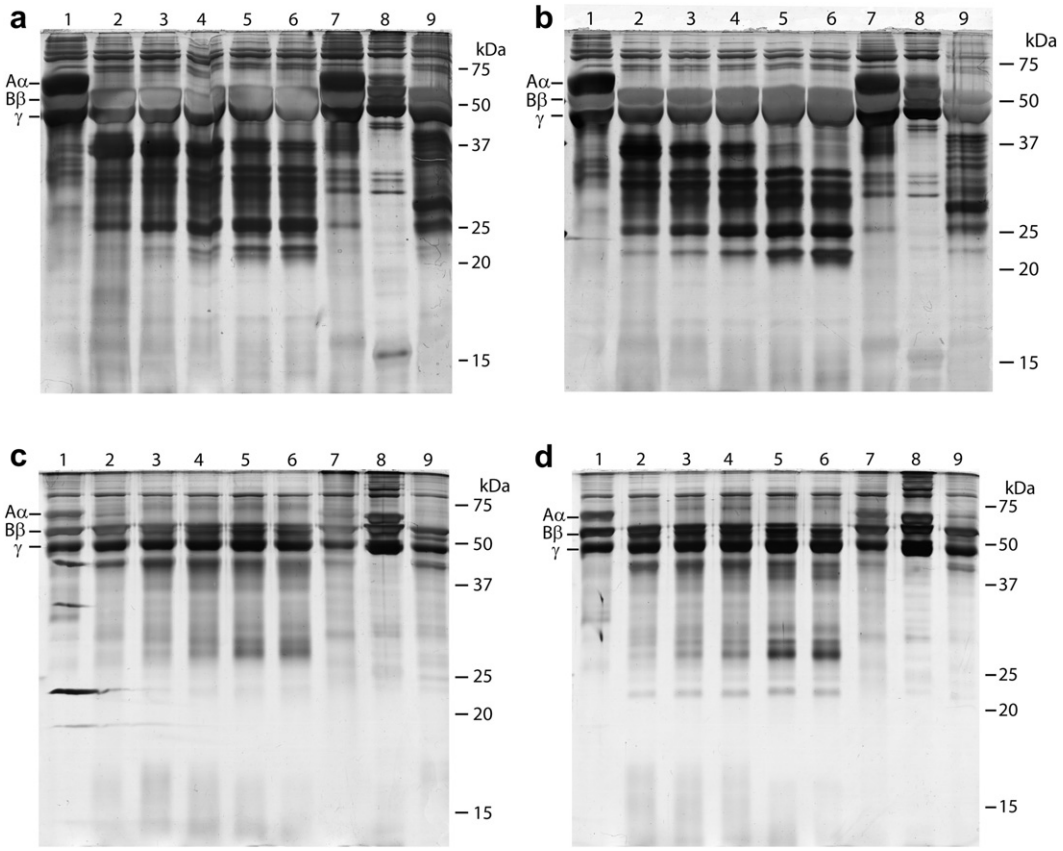

Fig. 5. Fibrinogenolytic activity of newborn (a, c) and adult (b, d) *B. jararaca* venom evaluated using rabbit (a, b) or bovine (c, d) fibrinogen. Fibrinogen hydrolysis was evaluated by SDS-PAGE (14% running gels), and thereafter gels were silver stained. Lanes 1 and 7 are controls of fibrinogen incubated in the absence of venom for 0 and 180 min, respectively; lanes 2, 3, 4, 5 and 6 are fibrinogen samples incubated with *B. jararaca* venom for 15, 30, 60, 120 and 180 min, respectively; lanes 8 and 9 – fibrinogen samples incubated for 180 min in the presence of 5 mM Na_2-EDTA and 8 mM PMSF, respectively.

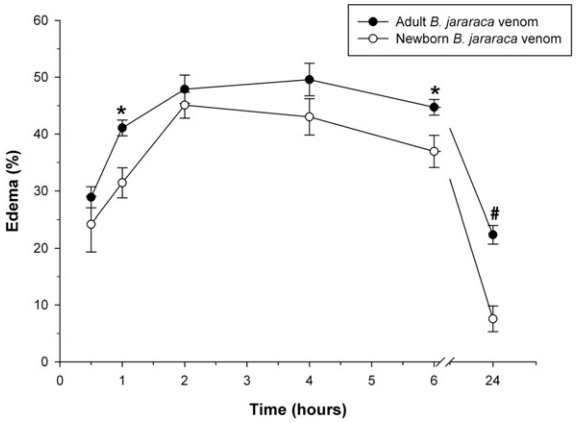

Fig. 6. Time-course of edematogenic activity induced by adult or newborn *B. jararaca* venom in mouse paws. Mice were injected with 1 μg of either venom into the subplantar surface of the right hind paw. Results were expressed as mean ± SEM of percentage increase in paw thickness ($n = 6$). Statistical difference between groups: $*p < 0.05$; $\#p < 0.001$.

3.9. Platelet aggregation

The newborn venom showed greater platelet aggregating activity than the adult venom (Fig. 7). ED_{50} values for platelet aggregation of washed human platelets induced by newborn and adult venoms were 2.46 μg/mL and 4.40 μg/mL (final concentrations), respectively.

3.10. Lethal activity

The adult venom was about two times more lethal in mice than the newborn venom. LD_{50} values for the adult venom (1.62 mg/kg, 95% confidence interval: 1.23–2.13 mg/kg) and the newborn venom (3.76 mg/kg, 95% confidence interval: 2.30–6.15 mg/kg) were significantly different ($p < 0.05$).

3.11. Neutralization of newborn and adult B. jararaca venoms by commercial Bothrops antivenin

Commercial *Bothrops* antivenin displayed much higher antibody titers by ELISA to the adult *B. jararaca* venom (titer 3,276,800) than to the newborn venom (titer 819,200), demonstrating that the commercial antivenin less effectively recognizes newborn venom proteins. This result was expected, since only venoms from adult specimens are used to compose the *Bothrops* sp. venom pool used to immunize horses. Likewise, when the efficacy of commercial antivenin was tested to neutralize the lethal activity of venoms in mice, the antivenin was demonstrated to be statistically less powerful (confidence limit 95%) to neutralize the newborn venom (141.92 μL/mg venom; 95% confidence interval: 124.98–161.16 μL/mg) than the adult venom (66.98 μL/mg venom; 95% confidence limit: 60.91–73.65 μL/mg). In an attempt to identify whether high concentrations of antibodies (1:1000) found in commercial antivenin could identify protein spots of newborn venom, a Western blotting was used (Fig. 8). Apparently, all spots present in both venoms were recognized by antivenin (Fig. 8 *cf*. Fig. 3).

Table 3
Hemorrhagic activity of newborn and adult *B. jararaca* pooled venoms.

		B. jararaca venom	
		Adult	Newborn
Minimum hemorrhagic dose (MHD) (μg venom/mice)		1.33	2.09
Paw hemorrhage (mg hemoglobin/g of paw tissue)	At 2 h	3.66 ± 0.55 ($n = 6$)	2.74 ± 0.86† ($n = 6$)
	At 24 h	10.00 ± 1.21 ($n = 6$)	6.53 ± 0.59* ($n = 6$)

Statistical difference between groups injected with adult and newborn *B. jararaca* venom: $\dagger p = 0.388$; $*p = 0.028$.

4. Discussion

As shown here, the chemical composition, biological activities and immunological properties of *B. jararaca* venom change during their ontogenetic development, and such changes certainly contribute to the diverse outcomes of bites by adult and newborn *B. jararaca* snakes. Newborn *B. jararaca* venom is remarkably more coagulant, and less hemorrhagic, inflammatory and lethal. Importantly, newborn venom is less efficiently neutralized by commercial *Bothrops* antivenin.

In regard to the chemical composition of adult and newborn *B. jararaca* venoms, no significant differences were noticed in the content of proteins and neutral hexoses. The content of sugars is proportional to the amount of glycoproteins in snake venoms (Oshima and Iwanaga, 1969; Aragón et al., 1977). In another study, Zelanis et al. (2010) also observed that the glycosylation level did not vary during ontogenetic development in *B. jararaca*. Glycosylation may prevent protein degradation inside venom gland in venoms with high proteolytic activity, since the content of neutral hexoses in *B. jararaca* venom was 3 times higher than those found in *Crotalus durissus terrificus*, whose venom do not exhibit strong proteolytic activity (Santoro et al., 1999). In effect, zinc levels in *B. jararaca* venoms, at least six times higher than those reported for the venom of *C. durissus* subspecies

Table 4
Coagulant and anticoagulant activities of adult and newborn *B. jararaca* pooled venoms. Minimum coagulant dose (MCD) was determined in rabbit, rat and human plasmas, and bovine fibrinogen.

	B. jararaca venom	
	Adult	Newborn
MCD -Plasma		
Rabbit	4.67[a]	0.16
Rat	12.98	4.29
Human	9.79	1.14
MCD -*Bovine fibrinogen*	102.18	133.67
Prothrombin activation (μmol *p*-nitroaniline/min/mg venom)	236.19 ± 5.24	8531.50 ± 380.83
Factor X activation (μmol *p*-nitroaniline/min/mg venom)	101.40 ± 7.07	3140.09 ± 724.07
Protein C activation (μmol *p*-nitroaniline/min/mg venom)	0.53 ± 0.40	22.75 ± 17.67

[a] MCD was expressed as μg venom/mL.

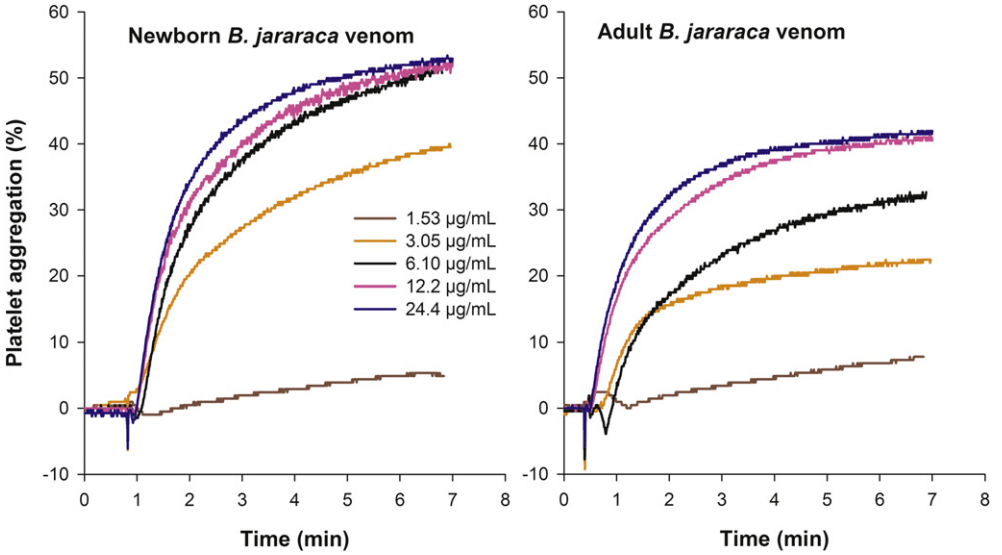

Fig. 7. Tracings of platelet aggregation from human washed platelets (300×10^9/L) stimulated by dilutions of either newborn or adult *B. jararaca* venoms. After 5 min the intensity of platelet aggregation was determined. Final venom concentrations in the suspension of washed platelets are shown.

(Santoro et al., 1999), likely reflect the higher content of metalloproteinases in *B. jararaca* venom. The total lipid content observed in the *B. jararaca* venom was lower than those reported in *C. durissus terrificus* venom (1%) (Devi, 1968); little is known about the role of lipids in venoms, but it has been shown that they are integral components of phosphodiesterases found in *Crotalus adamanteus* venom (Dolapchiev et al., 1980).

The increase in serum CK levels induced by the adult and newborn venoms may not be primarily attributed to PLA_2 with both catalytic and myotoxic activity, since the newborn venom showed a catalytic PLA_2 activity almost two times higher than that of the adult venom, but both venoms showed similar myotoxicity. Various Lys-49 PLA_2 isolated from *Bothrops* sp venoms do not show catalytic activity, but display high myotoxic and inflammatory activity, which could explain the discrepancy observed between myotoxic and catalytic PLA_2 activity in newborn and adult *B. jararaca* venoms (Gutiérrez and Lomonte, 1995). Compared to other *Bothrops* venoms that display high myotoxic activity, such as *Bothrops jararacussu*, *B. jararaca* venom has negligible levels of myotoxins (Moura-da-Silva et al., 1991), due to the low expression of PLA_2 in *B. jararaca* venom gland (Kashima et al., 2004; Cidade et al., 2006).

One- and two-dimensional electrophoreses revealed similarities in the composition of the venom pools studied, so that 61.6% of spots are common to both venoms. By one- and two-dimensional gel electrophoresis, the newborn venom was shown to contain more densely stained bands and spots of high molecular mass, in agreement with previous descriptions (Kamiguti, 1988; Furtado et al., 1991). The two-dimensional gel electrophoresis showed that these high molecular mass proteins of the newborn venom are acidic, likely part of the P-III class of metalloproteinases (Serrano and Maroun, 2005; Serrano et al., 2005; Guércio et al., 2006). Thus, *B. jararaca* venom undergoes changes in protein expression upon reaching adulthood, occurring (1) a reduction in the synthesis of acidic metalloproteinases, whose molecular mass corresponds to P-III metalloproteinases; (2) a dramatic increase in the expression of one spot with molecular mass and pI close to those of either jararhagin or bothropasin (Laing and Moura-da-Silva, 2005; Muniz et al., 2008); and (3) an augmented expression of basic proteins.

The newborn *B. jararaca* venom is less hemorrhagic both to mouse skin and footpads. Conversely, other *Bothrops* species show increased hemorrhagic activity in newborn venoms (Gutiérrez et al., 1980; Saldarriaga et al., 2003). Hemorrhage induced by *B. jararaca* venom is generally attributed to P-III metalloproteinases – e.g. jararhagin, bothropasin, and HF-3 (Mandelbaum and Assakura, 1988; Paine et al., 1992) –, since this class of metalloproteinases are potent hemorrhagins (Fox and Serrano, 2005). Reduced hemorrhagic activity in the newborn *B. jararaca* venom seems thereby to correlate with the fainting of the protein spot corresponding to both molecular mass (52 kDa) and pI (4.5) of jararhagin in newborn venom. The demonstration of a conspicuous area of lysis close to 50 kDa in gelatin zymography, which was remarkably more distinct in the adult venom, suggests that jararhagin is the main enzyme involved in the collagenolytic activity of the adult *B. jararaca* venom. The newborn venom had also a lower collagenolytic and fibrinogenolytic activity, in agreement with other reports demonstrating a weak proteolytic activity at this ontogenetic stage (Rosenfeld et al., 1959a; Kamiguti, 1988; Furtado et al., 1991). Marked inhibition of collagenolytic and fibrinogenolytic activities by incubation with EDTA and *o*-phenanthroline reveals the importance of metalloproteinases to protein hydrolysis in the adult and newborn crude venom.

The i.p. injection of *B. jararaca* venom in rat footpad causes a dose-dependent edema, which is primarily mediated by arachidonic acid metabolites generated by lipoxygenase and cyclooxygenase enzymes (Trebien and Calixto, 1989). Injection of adult and newborn *B. jararaca*

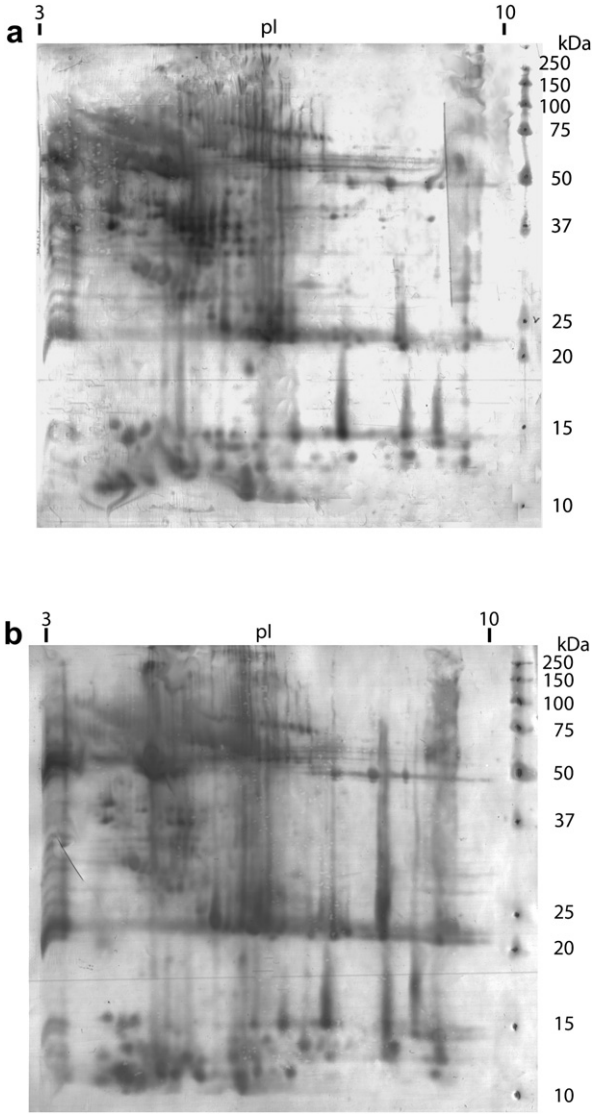

Fig. 8. Western blotting of newborn (a) and adult (b) and pooled *B. jararaca* venom. Venom proteins were subjected to two-dimensional electrophoresis (see item 2.5.2.), and blotted onto a nitrocellulose membrane. Membranes were then sequentially incubated with commercial antivenin and peroxidase-conjugated anti-horse IgG. The reaction as described previously (Pukac et al., 1997).

snake venom induced inflammatory edema in mouse footpads, but the edema induced by the adult venom was more intense. In opposition, adult *Bothrops atrox* and *Bothrops asper* venoms causes less intense edema than newborn venoms in mice (Chaves et al., 1992; Saldarriaga et al., 2003). The finding that the adult *B. jararaca* venom is more edematogenic may be correlated with its higher proteolytic activity, which may exert major tissue damage on tissues and, consequently, an augmented inflammatory response compared to that induced by the newborn venom. On the other hand, the higher L-amino oxidase and hyaluronidase activity in the newborn venom indicates that these two enzymatic activities do not seem to have a major contribution to *B. jararaca*-induced edema. Kamiguti (1988) also showed that edema induced in rats by the adult *B. jararaca* venom is more intense than that induced by the newborn venom, although the kinetics of edema formation in rats and mice is diverse. Despite the occurrence of eicosanoid generation in the inflammatory response induced by adult and newborn *B. jararaca* venoms in rats, the mediation of edema formation does not seem to be identical in both cases, since methylsergide could exclusively abrogate the edema induced by the adult venom (Kamiguti, 1988).

It is tempting to speculate that the shift that occurs in *B. jararaca* venom during its development is mainly due to differential expression of P-III metalloproteinases. High molecular mass spots in the newborn *B. jararaca* venom are distributed in the same region of *B. atrox* venom P-III metalloproteinases homologous to berythractivase (Guércio et al., 2006), a 78-kDa prothrombin activator from *Bothrops erythromelas* venom, which is devoid of hemorrhagic activity (Silva et al., 2003). As demonstrated here and elsewhere (Kamiguti, 1988; Furtado et al., 1991; Zelanis et al., 2010), newborn *B. jararaca* venom is more potent to activate prothrombin and factor X, and such ability is due to the higher activity of procoagulant metalloproteinases therein, since the metal chelators EDTA and EGTA abrogate such thrombin-generating activity (Santoro and Sano-Martins, 1993). The spot corresponding to the prothrombin activator isolated from *B. jararaca* venom (molecular mass of 22.8 kDa and pI of 8.3) (Berger et al., 2008) has a reduced expression in the newborn venom, suggesting that the enhanced ability of this venom to activate prothrombin is due to the differential expression of other prothrombin activators. Therefore, the more intense coagulant activity of the newborn venom may be attributed to the differential expression of metalloproteinases involved in the activation of prothrombin and factor X, and not to an increase in thrombin-like activity (serine proteinases), confirming previous results (Rosenfeld et al., 1959a; Kamiguti, 1988; Furtado et al., 1991). Although protein C activators have been described in other snake venoms (Stocker et al., 1986), this is the first report to show that *B. jararaca* venom also contains them. In agreement with the enhanced ability to activate prothrombin and factor X, newborn *B. jararaca* venom is also more potent to activate protein C, whose activation may also play a role in disturbing hemostasis by inactivating factors Va and VIIIa. Additionally, newborn *B. jararaca* venom has a higher platelet aggregating activity, theoretically rendering it more prone to induce platelet disturbances. However, human patients bitten by young *B. jararaca* snakes have higher platelet counts in comparison with those bitten by adult snakes (Santoro et al., 2008). Bites by newborn *B. jararaca* snakes may engender higher amounts of intravascular thrombin and/or meizothrombin, by means of greater activation of circulating factor II and X. In fact, victims of bites by young *B. jararaca* snakes manifest a higher incidence of clinical signs associated with augmented generation of intravascular thrombin (Sano-Martins et al., 2009), such as intravascular hemolysis and acute renal failure (Málaque et al., 2006; Novaes et al., 2006; Sano-Martins et al., 2009). Altogether, these pieces of evidence demonstrate that the hemostatic disturbance in patients bitten by young *B. jararaca* is more intense and may be more severe.

We show here that newborn *B. jararaca* venom is less lethal to mice, in agreement with other reports (Furtado et al., 1991; Andrade and Abe, 1999; Zelanis et al., 2010). *Bothrops* antivenin (Institute Butantan), used in the treatment of *B. jararaca* bites, is produced by hyperimmunization of horses with a venom pool containing 50% of *B. jararaca* venom, and 12.5% each of the following venoms: *Bothrops alternatus*, *B. jararacussu*, *Bothrops moojeni*, and *Bothrops neuwiedi* venoms (Cardoso et al., 2003). Such venom pool uses exclusively venoms obtained from adult snakes (personal communication from Dr. Kathleen F. Grego, Laboratory of Herpetology, Institute Butantan). When such antivenin was used in immunoneutralization tests, lower antibody titers to newborn *B. jararaca* venom were demonstrated, but low dilutions of antivenin could recognize antigens blotted from two-dimensional gels. These *in vitro* data demonstrate that commercial antivenin failed to recognize proteins present exclusively in newborn *B. jararaca* venom, and that higher amounts of antivenin are required to recognize proteins from the newborn venom. Furthermore, tests accomplished in mice to test the ability of commercial antivenin to neutralize the lethal activity of both venoms showed that the antivenin was two times less effective to neutralize newborn venom, even though this venom is two times less lethal than the adult venom. Kamiguti (1988) also showed that commercial antivenin was four times less efficient to neutralize the coagulant activity of newborn venom in comparison to the adult venom. Such pieces of evidence demonstrate that higher doses of antivenin should be used to treat patients bitten by newborn snakes if the same amount of venom was theoretically injected by adult and newborn snakes. The criterion currently adopted by the Ministry of Health in Brazil (Brasil, 2001) for serotherapy in victims of *Bothrops* spp bites does not take into consideration the severity of coagulopathy, but it recommending that additional doses of antivenin should be exclusively administered if local edema – which is not an important clinical manifestation in bites from young *B. jararaca* snakes (Rosenfeld et al., 1959b; Ribeiro and Jorge, 1990; Ribeiro et al., 2001) – becomes more severe. Thus, even evoking higher frequencies of hemostatic disturbances in victims (Ribeiro and Jorge, 1989; Sano-Martins et al., 2009), the severity of envenomation by newborn *B. jararaca* snakes will be invariably considered mild, since the local lesions are less severe in these patients. Consequently, even if the coagulopathy is more severe in these patients, the number of antivenin vials recommended for treatment of snakebites by young snakes will be that recommended for mild envenomation, i.e., the maximum of four vials. Inasmuch as the lethal and coagulant activities of newborn venom are not neutralized so efficiently by antivenin, a complete evaluation of hemostatic parameters, and not only of whole blood clotting time, should be employed in patients bitten by *B. jararaca* as a criterion to administer higher amounts of antivenin. Another alternative to treat patients more efficiently, using lower doses of antivenin, would be the incorporation of venoms from newborn and young snakes in the venom pool used for hyperimmunization of horses, so that antibodies specific to proteins exclusively present in young snakes would be raised.

In conclusion, our findings show that the venom undergoes important changes during the ontogenetic development of *B. jararaca*, especially in regard to the hemostatic, hemorrhagic, proteolytic, lethal and inflammatory activities. Our results also highlight the necessity to carry out a reassessment of clinical approaches currently used to administer antivenin to victims of young snakes. As shown herein, the toxicity of young *B. jararaca* snakes should not be underestimated by physicians.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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