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Systemic pathological alterations caused by *Philodryas patagoniensis* colubrid snake venom in rats

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Abstract

Very little is known about the systemic effects caused by *Philodryas patagoniensis* colubrid snake venom. In this work, this venom was tested for its ability to induce histopathological changes in rats after its intramuscular, subcutaneous or intravenous administration, by light microscopic examination of some organs (cerebellum, cerebrum, lung, liver, kidney and heart). Four rats were used for each dose of 0.23, 0.45 and 0.90 mg of venom in 0.3 ml of phosphate-buffered saline solution (pH 7.4). Aliquots of blood were withdrawn at different time intervals for enzymatic determination of alanine aminotransferase, aspartate aminotransferase and creatine kinase levels. After 2 h the animals were killed by an overdose of anesthetic, and samples of kidney, heart, liver, lung, cerebrum and cerebellum were taken to microscopic examination (hematoxylin and eosin stain). Histologically, no abnormality was observed in heart tissue, in none of the administration routes of the venom used. However, histological observations showed multifocal hemorrhage in cerebellum, cerebrum and lung sections, severe peritubular capillary congestion in kidney sections and hydropic degeneration in liver sections, when venom was administrated intravenously. The subcutaneous route showed similar results to the previous one, with the exception of cerebellar hemorrhage. Intramuscularly, neither cerebral nor cerebellar hemorrhage was observed. Plasma alanine aminotransferase and aspartate aminotransferase increased levels were demonstrated, mainly when venom was administered intravenously or subcutaneously. Our results suggest that *P. patagoniensis* venom induces moderate histopathological changes in vital organs of rats. These changes are initiated at early stages of the envenomation and may be associated with a behavioral or functional abnormality of those organs during envenoming.

Keywords: Colubridae; *Philodryas patagoniensis*; Venom; Systemic histopathological changes

1. Introduction

The composition of toxic oral secretions (venoms) from snakes of the family *Colubridae* are largely unknown, even though this exceptionally diverse family contains well over half of the described

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extant species of snakes, and perhaps half of these produce toxic secretions from the Duvernoy's gland (Gans, 1978; Underwood, 1979; Minton, 1990, 1996; Mackessy, 2002).

The toxic effects associated with bites from certain species of colubrids are the result of toxins secreted from the Duvernoy's gland (DG) (Weinstein and Kardong, 1994). However, not all colubrid snakes apparently possess DG and not all secretions from DG are toxic (Weinstein and Kardong, 1994). In those colubrids that produce toxic secretions the result of envenoming may be very mild to fatal depending on the snake, the victim and the amount of secretion injected into the victim (Kamiguti et al., 2000).

While many reports are found on venom constituents of many front-fanged species of snakes (e.g. *Viperidae* and *Elapidae*), very little has been done to elucidate the composition and biological activities of venoms of the polyphyletic family *Colubridae*, the world's largest snake family (Hill and Mackessy, 2000; Kamiguti et al., 2000; Mackessy, 2002). This is probably because these species are mainly rear- or mid-fanged which means that is not only more difficult for them to physically bite a human, but also because it is much harder to extract venom for research purposes (Hill and Mackessy, 2000; Kamiguti et al., 2000; Mackessy, 2002). Dangerously venomous colubrids are less commonly encountered, and serious bites typically require longer contact times, and thus they pose a minor problem to humans compared with the two major families of medically important land snakes, the *Viperidae* and the *Elapidae* (Kamiguti et al., 2000).

Most of the venomous colubrids may not produce venom capable of causing serious damage to humans, but at least five species (*Dispholidus typus*, *Thelotornis capensis*, *Rhabdophis tigrinus*, *Philodryas olfersii* and *Tachymenis peruviana*) have caused fatal human envenomations (Pope, 1958; FitzSimons and Smith, 1958; Mittleman and Goris, 1976; Ogawa and Sawai, 1986; Salomão and Di-Bernardo, 1995; Vellard, 1955; Hill and Mackessy, 2000; Mackessy, 2002). Other species of rear-fanged colubrids have not caused human deaths but produce venoms with some characteristics similar to front-fanged snakes (Hill and Mackessy, 2000; Mackessy, 2002).

Philodryas is a genus of rear-fanged colubrid snakes, which is found in South America, from Amazonas to Patagonia (Assakura et al., 1992, 1994). Bites by species of *Philodryas* have been reported from Argentina (Orduna et al., 1994), Brazil (Nickerson and Henderson, 1976; Silva and Buononato, 1983/84; Nishioka and Silveira, 1994; Araújo and dos Santos, 1997) and Chile (Schenone et al., 1954; Schenone and Reyes, 1965). Most envenomations caused by these colubrid snakes are mild, involving mainly local pain, edema and ecchymosis (Fowler and Salomão, 1994; Carvalho and Nogueira, 1998; Ribeiro et al., 1999). Systemic envenomation is rare, but a general malaise was reported to be common in bites by *P. chamissonis* (Schenone and Reyes, 1965). Ribeiro et al. (1999) reported one case of envenomation by *P. olfersii* in a 2-year-old child which resulted in nausea and vomiting. Furthermore, Orduna et al. (1994) reported an altered prothrombin clotting time in a patient bitten by *P. olfersii*. Moreover, one fatality suspected to have been caused by a colubrid (*P. olfersii* in Brazil) has been recorded (Salomão and Di-Bernardo, 1995). Hence, the conclusion of some that the effects of envenomation by species of *Philodryas* are generally mild (Fowler and Salomão, 1994; Carvalho and Nogueira, 1998; Ribeiro et al., 1999), perhaps not all would subscribe to this view (Warrell, 1996).

The properties of crude venoms from *P. olfersii* and *P. patagoniensis* have been studied extensively (Assakura et al., 1992, 1994; Acosta de Pérez et al., 2003; Acosta et al., 2003; Peichoto et al., 2004, 2005). At present, the local effects resulting from envenoming by these colubrid snakes are well known (Assakura et al., 1992; Acosta de Pérez et al., 2003; Acosta et al., 2003; Peichoto et al., 2004). Nonetheless, very little is known about systemic effects capable of being induced by these venoms (Assakura et al., 1994; Peichoto et al., 2005).

Philodryas patagoniensis, an opistoglyphous colubrid snake with a well-developed DG connected with an enlarged grooved rear maxillary tooth, is found in South America: Argentina, Bolivia, Brazil, Paraguay and Uruguay (Peters and Orejas-Miranda, 1970; Rocha and Molina, 1987). Human envenoming by this colubrid snake, although rare and not yet reported to be lethal, leads to pain, swelling, warmth and ecchymotic lesions on the bitten limb, which bear a striking resemblance to the signs and symptoms of bothropic envenoming (Nishioka and Silveira, 1994; Araújo and dos Santos, 1997). In agreement with the above-mentioned, our recent studies about *P. patagoniensis* venom have demonstrated that the local tissue damage is characterized by hemorrhage, edema, myonecrosis and dermonecrosis (Acosta et al., 2003; Peichoto et al., 2004). The pathogenesis of

venom-induced local effects is rather complex, mainly involving the action of metalloproteinases (Acosta et al., 2003; Peichoto et al., 2004), although it is not discarded the combined action of metalloproteinases and other venom components. With the exception of a previous work (Peichoto et al., 2005), in which we demonstrated that *P. patagoniensis* venom induces an *in vivo* decrease of rat plasma fibrinogen, very little is known about the systemic effects caused by this venom. In this paper, we present the first widely available report on the histopathological changes induced by the venom of *P. patagoniensis*, a colubrid snake found in northeastern Argentina, in vital organs of rats such as cerebrum, cerebellum, lung, liver and kidney, via three different administration routes of the venom.

2. Materials and methods

2.1. Venom of P. patagoniensis

Pooled venom was obtained from several adult specimens of *P. patagoniensis* captured in northeastern Argentina, and then maintained at the serpentarium of the local Zoo, Corrientes, Argentina. Specimens were milked by introducing a 100 μl micropipette under each fang, according to the procedure described by Ferlan et al. (1983). Venom was lyophilized and thereafter kept frozen at −20 °C. When required, venom was dissolved in phosphate buffered saline solution, pH 7.4 (PBS). The small amount of insoluble material was removed by centrifugation, and the clear supernatant was applied for studies.

2.2. Animals

Adult male Sprague–Dawley rats (180–200 g body weight) were supplied by the Animal House, School of Veterinary Sciences, University of Northeastern Argentina. Food (chow rat diet) was withdrawn 12–14 h before the experiment, but the animals had free access to water. Temperature in the animal room was 23 ± 2 °C and the relative humidity was between 35% and 65%. Lights in the animal room were on from 6 a.m. to 6 p.m. Four animals per group were used, as stated in each experimental design. All experiments followed the ethical standards for animal experiments in toxinological research recommended by the International Society of Toxinology (Meier et al., 1993).

2.3. Histological characterization of systemic pathological effects induced by P. patagoniensis venom

Venom was inoculated into iliac vein, subcutaneous tissue or gastrocnemius muscle of rats. Four rats were used for each dose of 0.23, 0.45 and 0.90 mg of venom in 0.3 ml of PBS, and for each time interval (in cases of s.c. and i.m. injection). The doses were chosen based on an estimate of the amount of venom that would be able to produce lesions without causing the death of the animals. Control rats were inoculated with 0.3 ml of PBS.

The method described by Assakura et al. (1994) was used for i.v. administration of the venom. Rats were previously anesthetized with i.p. injection of chloral hydrate (300 mg/kg) and heparinized with 1000 IU/kg, i.v. Aliquots (1 ml) of blood were withdrawn 1 and 2 h after venom inoculation. Blood control (1 ml) was removed 15 min before inoculation. Administration of venom and removal of blood samples were made through a polyethylene catheter introduced into the iliac vein. In order to maintain a constant blood volume, each blood sample collected was replaced by inoculating an equivalent volume of 0.9% NaCl.

Plasma was obtained for enzymatic determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatine kinase (CK) levels using Ultraviolet Kinetic kits (Sigma). The ALT activity was expressed in units per liter, with one unit defined as the amount of enzyme that catalyzes the conversion of 1.0 micromole of L-alanine into 2-Oxoglutarate per min at the conditions of the assay. The AST activity was expressed in units per liter, with one unit defined as the amount of enzyme that catalyzes the conversion of 1.0 micromole of L-aspartate into 2-Oxoglutarate per min at the conditions of the assay. The CK activity was expressed in units per liter, with one unit defined as the amount of enzyme that catalyzes the conversion of 1.0 micromole of phosphocreatine into creatine per min at the conditions of the assay.

After 2 h the animals were killed by an overdose of anesthetic, and samples of kidney, heart, liver, lung, cerebrum and cerebellum were taken and fixed with Bouin solution for 24–48 h. Thereafter, the tissue samples were dehydrated in a graded alcohol series and embedded in paraffin. Five μm sections were cut in a microtome and stained with hematoxylin and eosin to be examined under a light

microscope. The control samples were processed as described above.

2.4. Statistical analysis

The results of the enzymatic determination of plasma AST and ALT levels were expressed as the mean ± standard deviation (SD). Differences between groups were compared using one-way analysis of variance (ANOVA) followed by the Dunnett test. A value of $p < 0.05$ indicated significance.

3. Results and discussion

In this work, the venom of *P. patagoniensis* was tested for its ability to induce histopathological changes in rats after its administration via three different routes, by light microscopic examination of some organs (cerebellum, cerebrum, lung, liver, kidney and heart).

Two hours after i.v. administration of 0.23, 0.45 and 0.90 mg of *P. patagoniensis* venom, light microscopic examination of cerebellum sections showed moderate but evident multifocal hemorrhage in the white matter (Fig. 1), whereas no evidence of hemorrhage was noticed in granular, molecular and Purkinje cell layers of the cerebellar cortex (gray matter). Cerebrum sections also showed multiple small foci of hemorrhage and congestion of some blood vessels, mainly in the prelimbic and infralimbic areas of the prefrontal cortex (Fig. 1). When injected subcutaneously, venom only induced multifocal hemorrhage in the parietal cortex of the cerebrum. Intramuscularly, neither cerebral nor cerebellar hemorrhage was observed.

Besides multifocal hemorrhage which was evidenced by the presence of abundant erythrocytes in bronchiolar and alveolar spaces, lung sections demonstrated mixed inflammatory infiltrate of polymorphonuclear and mononuclear cells that dilated the alveolar septa, and intense diffuse congestion of pulmonary parenchyma (Fig. 1). The venom-induced pulmonary hemorrhage was evident via the three administration routes and with the three venom doses assayed.

These results agree with a previous study (Acosta et al., 2003) in which we demonstrated that *P. patagoniensis* venom possesses conspicuous proteolytic activity. Therefore, the capacity of this venom to induce systemic hemorrhage depends on its intrinsic hemorrhagic potency, which very likely depends on its ability to hydrolyze peptide bonds of basal lamina components that are critical for the stability of this extracellular matrix scaffold (Bjarnason and Fox, 1994; Baramova et al., 1990a, b, 1991; Gutiérrez and Rucavado, 2000; Gutiérrez et al., 2005). Furthermore, it is very likely that this venom contains hemorrhagic proteinases that are resistant to the inhibitory action of plasma proteinase inhibitors, mainly α-macroglobulins (Baramova et al., 1990a, b; Kamiguti et al., 1994; Anai et al., 1998; Gutiérrez et al., 2005). The latter will be deciphered when purification and characterization of components of *P. patagoniensis* venom are carried out. Kamiguti et al. (2000) have already demonstrated, by immunochemical and mass spectrometric analyses, that the venom of the African colubrid *Dispholidus typus* contains P-III metalloproteinases that cross-react with jararhagin, a potent hemorrhagic toxin from *Bothrops jararaca* venom capable of inducing systemic hemorrhage.

Furthermore, it has been reported that *P. patagoniensis* venom contains fibrinogenolytic enzymes (Peichoto et al., 2005), which induce an *in vivo* decrease of rat plasma fibrinogen. Although fibrinogenolytic activity does not appear to be essential for inducing hemorrhage, it may play an important role by interfering with blood coagulation and the formation of hemostatic plug (Markland, 1998; Gutiérrez and Rucavado, 2000). As *P. patagoniensis* venom is devoid of coagulant activity (Peichoto et al., 2005), hemorrhage is not associated with consumption of clotting factors. It is interesting to note that hemorrhage in brain and lungs, besides other organs, is commonly found in patients bitten by viperid snakes (Efrati, 1979) whose venoms have both hemorrhagic and coagulant activities.

A possible explanation for the occurrence of hemorrhagic manifestations in particular organs such as cerebellum, cerebrum and lungs, could have to do with the predominant distribution of *P. patagoniensis* venom to these organs. Alternatively, cerebellum, cerebrum and lung microvasculature may be more susceptible to the action of this venom than microvessels from other organs. These hypotheses require further investigation.

Microscopic examination of liver sections showed diffuse congestion of blood vessels and sinusoidal dilatation, which were evident via the three different administration routes of the venom. A few necrotic hepatocytes could be observed around central veins, but only when the highest dose of venom was inoculated intravenously. Hydropic degeneration

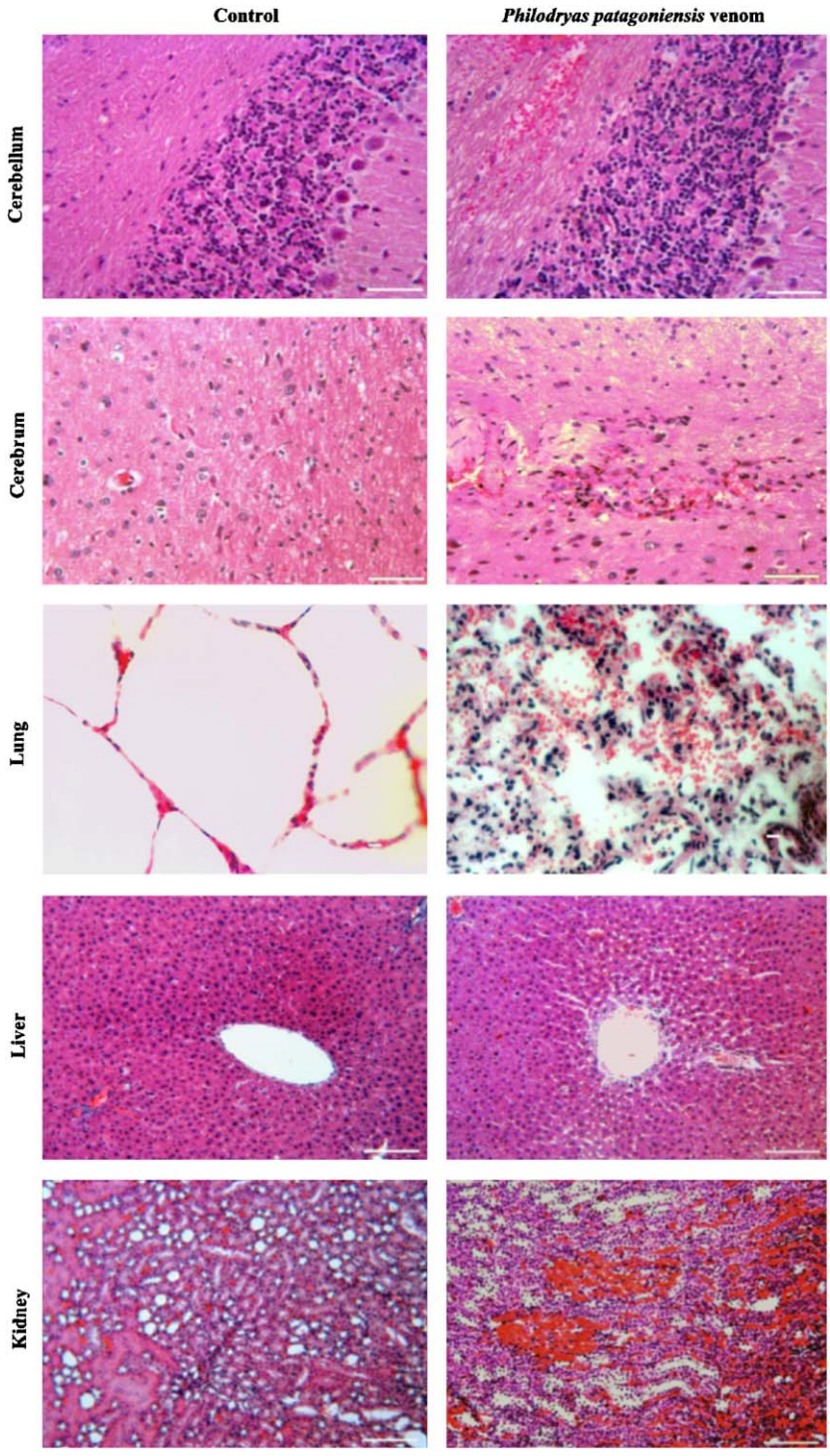

Fig. 1. Light micrographs showing the histopathological changes induced by 0.90 mg of *Philodryas patagoniensis* venom in vital organs of rats after 2 h of its i.v. inoculation. Sections were stained with hematoxylin and eosin. *Cerebellum*: note hemorrhage, revealed by the presence of erythrocytes in the white matter. Scale bar, 50 μm. *Cerebrum*: note the presence of hemorrhage in the internal granular layer of the prefrontal cortex. Scale bar, 50 μm. *Lung*: note extravasation of erythrocytes in some alveolar spaces and mixed inflammatory infiltrate of polymorphonuclear and mononuclear cells in the alveolar septa. Scale bar, 10 μm. *Liver*: note squashed hepatocytes and dilated sinusoids around a central vein. Scale bar, 100 μm. *Kidney*: note severe peritubular capillary congestion and tubular dilatation. Scale bar, 100 μm.

was particularly noted when venom was also administered intravenously. Hepatocytes with hydropic changes are swollen edematous hepatocytes with clumped cytoplasm and large clear spaces, which were mainly observed in centrilobular region (Fig. 1). The s.c. and i.m. routes showed less and no degeneration of hepatocytes, respectively. Unlikeness of what was observed with *P. patagoniensis* venom via the i.m. route, diffuse hydropic degeneration has been reported in rats i.m. injected with 8 mg/ml of *Bothrops alternatus* venom (Teibler et al., 1999), a higher dose than that used in this work.

ALT and AST increased levels in plasma samples from rats i.v. inoculated with *P. patagoniensis* venom were demonstrated (Fig. 2(A) and (B)). These increases were proportional to both the dose of venom and the time lapsed after its inoculation, and they were detected not only by the i.v. route but also by the s.c. one (data not shown). No enzymatic increase was observed via the i.m. route.

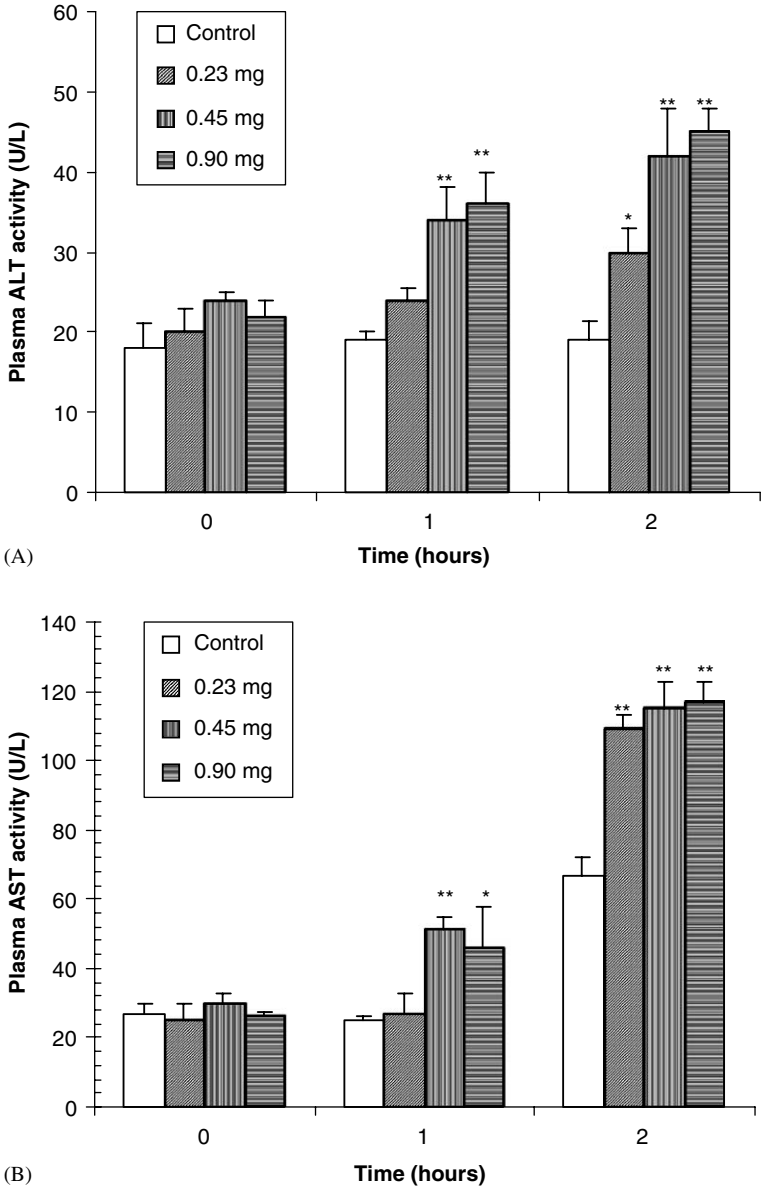

Fig. 2. Changes in rat plasma AST and ALT levels after i.v. inoculation of three different doses of *Philodryas patagoniensis* venom. (A) Plasma ALT activity versus time. (B) Plasma AST activity versus time. Activities were expressed in units/L and results were presented as mean ± SD ($n = 4$). *$p < 0.05$ and **$p < 0.01$ when compared to values of control rats.

ALT is an enzyme that is found primarily in the liver. Therefore, the mild increase of this enzyme detected in this study could be attributed to its release into the bloodstream as the result of liver damage caused by the venom. Simultaneously, a moderate increase of AST was observed, which would confirm the hepatic lesion. AST is an enzyme that is found in red blood cells, liver and heart cells, and skeletal muscle tissue. Therefore, the AST is less liver-specific than the ALT. For this reason, it is important to take into account that elevations of AST may also be seen in acute muscle injury (either cardiac or skeletal muscle). Mild elevations of CK were observed in plasma samples (1 and 2 h) from all i.v. inoculated rats, even those not injected with the venom (data not shown). As the heart of all the rats, even those inoculated with the venom, did not show any histopathological change, we can infer that the elevations of CK observed in all i.v. injected animals were only due to skeletal muscle damage. It was likely due to the use of chloral hydrate anesthetic, which is known to cause rhabdomyolysis (Coco and Klasner, 2004). Therefore, it would explain the mild elevation of plasma AST activity observed in control samples 2 h after i.v. administration of PBS (Fig. 2(B)).

From the above results, it is obvious that *P. patagoniensis* venom exhibits a hepatotoxic action reflected by alterations in both histological and enzymatic patterns of the hepatic tissue, as many viperid snake venoms do (Teibler et al., 1999; Aznaurian and Amiryan, 2006).

Intravenously, light microscopic examination of kidney sections showed severe diffuse peritubular capillary congestion (Fig. 1) and tubular dilatation; the latter was mainly observed in medullar zone. Intense congestion of blood vessels both in cortex and medulla was also observed when venom was administered intramuscularly or subcutaneously. By the latter, multifocal cloudy swelling of the kidney tubules was noted, particularly in the transition zone of cortex and medulla. No histological change was noted in heart sections under light microscope, in none of the administration routes of the venom used. It is interesting to note that hemorrhage in kidney tubules and myocardium is commonly found in patients bitten by viperid snakes (Efrati, 1979). Several workers have attributed the hemorrhagic activity to proteolytic activity of viperid venoms (Maeno et al., 1959; Kochwa et al., 1960; Ohsaka, 1960; Ohsaka et al., 1966; Iwanaga et al., 1965). However, the presence of hemorrhagic toxins devoid of proteolytic activities (towards conventional substrates) (Omori et al., 1964; Toom et al., 1969) and phospholipase A_2 enzymes inducing hemorrhage by themselves (Vishwanath et al., 1988), have also been reported. Therefore, we suggest that the synergistic action of proteolytic, coagulant and phospholipase A_2 enzymes would be involved in the development of not only hemorrhage but also any kind of deleterious effect on kidney and heart of patients bitten by viperid snakes (Boer-Lima et al., 1999). On the contrary, proteases would be mainly involved in effects caused by *P. patagoniensis* envenomation since this colubrid venom is devoid of coagulant and phospholipase A_2 activities (Acosta et al., 2003; Peichoto et al., 2004, 2005).

In conclusion, our results suggest that the venom of *P. patagoniensis* induces moderate histopathological changes in vital organs of rats such as cerebellum, cerebrum, lungs, liver and kidneys, after its i.v. inoculation. By subcutaneous and intramuscular routes, the venom induces moderate, mild or even no tissue lesion at all, depending on target organ. These changes are initiated at early stages of the envenomation and may be associated with a behavioral or functional abnormality of those organs during envenoming. Moreover, these damages may lead to permanent sequelae. As considerable caution should be exercised in extrapolating experimental studies in animals to human envenomation, it would be interesting to determine whether *P. patagoniensis* venom acts similarly in human victims. Some of our clinical observations (persistent and severe dizziness presented in victims bitten by this colubrid snake) suggest that this venom would induce not only local damage but also systemic pathological effects in humans. Discrete headache has been reported following envenomation by *P. chamissonis* (Schenone et al., 1954). Silva and Buononato (1983/84) reported the case of an individual bitten by *P. olfersii* who still complained of muscle weakness 14 days after the bite; however, no explanation was given to this point. It is curious that this weakness was reported in the legs, a site considerably distant from the bite site in the left forearm. A neurotoxic effect would be expected which is in agreement with recent findings on the proteomics of some colubrid venoms that have demonstrated the presence of proteins with the scaffold of 'three-finger neurotoxin' (Fry et al., 2003a, b).

Despite the lack of systemic alterations in clinical reports involving *P. patagoniensis*, caution should

be exercised when manipulating this colubrid snake, and bitten people should be closely observed for the potential development of systemic effects, since these effects have been described in bites by other species of *Philodryas* (Schenone et al., 1954; Schenone and Reyes, 1965; Silva and Buononato, 1983/84; Ribeiro et al., 1999; Orduna et al., 1994) and in experimental animals (this work). From the latter, it appears that in an actual human snakebite case, *P. patagoniensis* venom would be able to induce a systemic effect even when not injected via the intravenous route.

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