Systemic oxidative stress in victims of Bothrops snakebites

Juliana de Ornellas Strapazzona, Eduardo Benedetti Parisotto, Ana Maria Moratelli, Thais Regina Garlet, Juliana Bastos, Ivan Ricardo Zimermann, Marlene Zanin, Rodrigo Fagundes, Manuel Rosa de Oliveira Lino, Tânia Silvia Fröde, Danilo Wilhelm Filho

A R T I C L E   I N F O

Article history:
Received 8 August 2014
Received in revised form 13 November 2014
Accepted 19 November 2014
Available online 1 December 2014

Keywords:
Snakebites
Bothrops
Ophidic accidents
Inflammation
Oxidative stress
Antioxidants

A B S T R A C T

The genus Bothrops is responsible for approximately 90% of snakebites in Brazil. In the present study biomarkers of oxidative stress (OS) were evaluated in the blood of victims of snakebites from Bothrops jararaca and Bothrops jararacussu. Patient monitoring started from the emergency entrance at the hospital up to 30 days, groups divided as follows: time 0 (t₀), 24 hours (t₂₄h), 7 days (t₇d) and 30 days (t₃₀d). The activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), and myeloperoxidase (MPO), as well as the contents of reduced glutathione (GSH), vitamin E, lipid peroxidation (TBARS), protein carbonyls (PC) were examined in blood. Initial determinations revealed increased CAT, GR and GPx activities and decreased SOD and GST activities together with the depletion of GSH contents, while markers of oxidative damage showed increased TBARS levels and decreased PC concentrations in victims of snakebite compared to controls (blood donors). Regarding the temporal effect, no statistical differences among the groups were detected for the distinct parameters analyzed. The responses obtained in OS biomarkers in victims of snakebite compared to healthy subjects indicate that Bothrops envenomation promoted a pronounced and persistent systemic OS in the blood of those subjects.

© 2014 Faculty of Health and Social Studies, University of South Bohemia in Ceske Budejovice. Published by Elsevier Sp. z o.o. All rights reserved.
25,000 snakebites each year, while the genus Bothrops is responsible for approximately 90% of such accidents (Bernarde, 2014). The venom of Bothrops cause local intense pain, bleeding, tissue necrosis and inflammation, consisting of a complex variety of toxic components that act especially on the haemostatic system including platelet aggregation and induction of apoptosis, hemorrhage and cytotoxicity (Oliveira et al., 2008; Izidoro et al., 2014). A complex mixture of toxic proteins and enzymes involved in the inflammatory process often results in a chronic response, where metalloproteinases, anticoagulants, phospholipases, L-amino acid oxidases (LAAOs) and myotoxins have an important role in the action of the venom (Clissa et al., 2001; Izidoro et al., 2014).

As a consequence, antioxidant defenses are likely to be compromised by oxidative stress, as indicated by the single reference available on Bothrops snakebite (Cardoso et al., 2003). This inflammatory reaction results from a large influx of leukocytes, cytokine release, metabolites of nitrogen and also overgeneration of reactive oxygen species (ROS) (Petricevich et al., 2000; Zamuner et al., 2001). Furthermore, LAAOs contributes to oxidative stress through the generation of hydrogen peroxide (H₂O₂) and superoxide anion (Izidoro et al., 2014) two important precursors of hydroxyl radical (·OH) the most reactive and deleterious ROS (Halliwell and Gutteridge, 2007).

Intermediates of oxidative cellular metabolism, ROS are physiologically formed mainly by partial reduction of oxygen to water, which occurs by successive reactions in the chain of mitochondrial electron transport (Halliwell and Gutteridge, 2007). Therefore, ROS are able to react with important cellular components, oxidizing biomolecules such as amino acid residues, proteins, lipids and DNA (Moreno et al., 1980; Halliwell and Gutteridge, 2007). The imbalance between the mechanisms that cause oxidative damage and cellular antioxidant defenses present in aerobic organisms causes a variety of physiological changes collectively called oxidative stress (Sies, 1991). To minimize the deleterious effects associated with the constant ROS generation, all aerobic organisms have been endowed over evolutionary time with a panoply of different enzymatic and non-enzymatic antioxidant defenses (Halliwell and Gutteridge, 2007).

In the present study the blood oxidative stress was measured in victims of Bothrops snakebite and compared to healthy subjects, in order to gain a better understanding of this envenoming process.

Materials and methods

Patients

After hospitalization caused by snakebite, patients were treated in the Toxicological Information Center of the University Hospital Polyclinic Ernan de São Thiago (CIT/HU/UFSC, Florianópolis, south Brazil). This study involves two snake species found in south Brazil, Bothrops jararaca and Bothrops jararacussu, which are not so closely related in their phylogenetic positions (Wüster et al., 2002). Because only these two Bothrops species occurs in the region where the patients were victims of the snakebites, and also considering the circumstances and the difficulty related to undoubted species identification by the victims, in the present study only the genus taxonomic level was considered. After confirming the diagnosis by clinical evaluation, blood samples were immediately drawn from the cubital vein at the hospital emergency unit (t₀) and also at 1 (t₂₄h), 7 (t₇d) and 30 (t₃₀d) days after the snakebite. As exclusion criteria, patients with cognitive impairment or clinical consequences that would preclude the provision of data, or other reason to abandon the study, were not considered. Samples were obtained from 20 patients victims of snakebite, being 19 samples from Bothrops spp. and one from Micrurus sp. collected at time 0 (t₀), at admission in CIT/HU/UFSC; within 24 h (t₂₄h) 16 samples (3 left the hospital before 24 h from the snakebite and did not return); after seven days (t₇d) 14 samples (2 additional patients did not return after 1 week from the snakebite); and after 30 days (t₃₀d) 10 samples (4 additional patients did not return after 1 month from the snakebite) were collected. No further sample was excluded and all patients signed an informed consent form. As a control group, samples were collected from 22 patients randomly recruited from the Blood Bank of the same hospital (HU/UFSC).

Sample preparation

A volume of 15 mL of blood was collected from patients and the samples once processed and stored in liquid nitrogen, were used only for conducting the current analysis and afterwards were discarded. After intravenously collection into a tube containing heparin (or without heparin for obtaining serum), a whole blood fraction was immediately separated and precipitated with trichloroacetic acid (TCA) 12% (1:4, v:v), and stored in liquid nitrogen until the analysis of GSH. Separation of red blood cells and plasma was performed by a rapid centrifugation (3000 g for 3 min) of whole blood, and the corresponding aliquots were immediately stored in liquid nitrogen until analysis. To promote hemolysis distilled water (1:4, v:v) was added to the cells and stored in liquid nitrogen.

Antioxidant enzyme assays

CAT activity was analyzed at 240 nm according to Aebi (1984), which is based on the speed of H₂O₂ degradation. SOD activity was measured at 480 nm according to Misra and Fridovich (1972), modified by Boveris et al. (1983), by the oxidation of epinephrine, which produces superoxide anion and a pink chromophore. GPx determination was carried out by the method of Flohé and Gunzler (1984), measuring the rate of oxidation of NADPH at 340 nm. For GR measurements the method of Calberg and Mannervik (1985) was used, which checks at 340 nm the rate of oxidation of NADPH due to the formation of GSH from GSSG by the intervention of GR present in the assay. GST activity was measured at 340 nm using CDNB as substrate and GSH 0.1 M, according to Habig et al. (1976). All enzymatic determinations were carried out in duplicate.

Glutathione assay

Whole blood levels of GSH were measured in duplicate at 412 nm in acid extracts (TCA 12%, 1:4, v:v) obtained immediately after blood collection, by the method of Beutler et al.
Vitamin E evaluation

Determination of vitamin E in plasma was carried out by high performance liquid chromatography (HPLC) with UV detection at 292 nm (Nicoletti et al., 2001). An aliquot of 100 μL of plasma was added to 100 μL of ethanol and vortexed for 10 s and added to 100 μL of hexane, and again vortexed for 45 s. After centrifugation at 8000 g for 5 min, 75 μL of the supernatant (hexane) was transferred to an microtube and the hexane was evaporated by nitrogen flow, and 125 μL of diethylether and 37.5 μL of methanol were added and this mixture was injected in the HPLC device. Isocritical elution was carried out with methanol (100%) using a flow rate of 1 mL min⁻¹. Samples were measured in duplicate and the plasma concentration of α-tocoferol was determined through a standard curve and expressed as μmol vitamin E mL⁻¹.

Liperoxidation (TBARS levels) evaluation

The assessment of endogenous lipid peroxidation was performed in triplicate through the detection at 535 nm of substances that react with the thiobarbituric acid (TBARS), particularly its major product malondialdehyde (MDA), which produces a pink Schiff base (Bird and Draper, 1984).

Protein carbonyl contents

The oxidative damage to proteins was measured at 360 nm by contents of protein carbonyls according to Levine et al. (1990). The total protein concentration was determined by the biuret technique using an Analiza kit (São Paulo, Brazil), and total protein content was expressed in g dL⁻¹.

Myeloperoxidase assay

Samples were thawed at room temperature and 20 μL were transferred to buckets and the biochemical reaction initiated with the addition of 150 μL of medium buffer (165 μL of o-dianisidine 2HCl, 50 μL of H₂O₂ 30%, distilled H₂O and NaH₂PO₄ 50 mM). After 15 min of incubation at room temperature, the enzymatic reaction was stopped by addition of 15 μL of sodium azide 1% (Rao et al., 1993). After incubation for 10 min at room temperature the optical density was measured at 450 nm in a ELISA reader (Organon-Tecknica, Roseland, NJ, USA), and compared to standard curves with known concentrations of MPO (0.7–140 mU mL⁻¹). Myeloperoxidase (MPO) values were expressed as mU mL⁻¹.

Chlorazol black, which represents approximately 95% of non-protein small thiols.

Results

Within the first day (t₀, time zero) and also after 1, 7 and 30 days of the Bothrops snakebite, victims of snakebite showed SOD activity about one third lower (≥100 USOD mL⁻¹; statistically significant) compared to those (≥230 USOD mL⁻¹) found in healthy individuals (controls or blood donors) (Fig. 1A). On the contrary, CAT activity in the hemolysates of victims of snakebite showed about 14 μmol min⁻¹ mL⁻¹ (t₀), values that were approximately twice those showed by controls (≥7 μmol min⁻¹ mL⁻¹), which were kept in the same range during the first day of hospitalization and after 7 and 30 days of the snakebite (Fig. 1B). GPx activity showed a profile very similar to that of catalase, with significant differences at the time of admission (t₀; statistically significant) and 1, 7 and 30 days (statistically significant) after the snakebite compared to controls (Fig. 1C). Although GR activity in patients examined at all time intervals showed higher activity (≥1 μmol min⁻¹ mL⁻¹) compared to individuals from the control group, such differences were not statistically different (Fig. 1D). On the other hand, at all collection times GST activity was remarkably decreased (statistically significant; all values below 0.2 μmol min⁻¹ mL⁻¹) compared to subjects form the control group (≥1.0 μmol min⁻¹ mL⁻¹; Fig. 1E). No significant differences were detected for the activity of MPO comparing both groups (Fig. 1F). In addition, no significant differences were detected among the different collection times of blood samples for any of the enzymatic activities examined.

Compared to individuals from the control group, patients of snakebite at all times showed a highly significant decrease (≥60%; statistically significant) for GSH contents in whole blood, while no significant differences were detected among the different collection times (Fig. 2A). A similar profile was obtained for vitamin E in victims of Bothrops snakebite who showed an approximately half decrease in plasma concentrations since the first day and along the other monitored periods (Fig. 2B).

Curiously, regarding the markers of oxidative damage measured in the present study, plasma levels of lipid peroxidation and protein carbonyls, both parameters did not show statistical differences, neither when compared to controls nor among the distinct periods of time examined (Fig. 2C and D).

Discussion

After the Bothrops snakebite SOD activity was greatly diminished comparing with the values found in individuals from the control group (blood bank). This response could be associated with the hemorrhagic process caused by the venom, resulting in hemolytic processes of thermal injury to human red blood cells, thereby decreasing SOD activity (Ferreira and Matsubara, 1997). Accordingly, it was also reported that increased proteolytic activity resulted in a decrease of cellular SOD
activity due to removal of the SOD heparin-binding domain caused by proteolysis (Oury et al., 2002). In addition, Ueda et al. (2008) showed that pulmonary extracellular SOD levels were decreased during systemic inflammation, which could be related to the proteolytic action of MMPs present in the venom of Bothrops. Contrary to the response showed by SOD, CAT activity showed a significant increase in comparison with controls, especially for periods close to the snakebite. This enhanced CAT activity might be related to increased generation of H$_2$O$_2$, the specific substrate of this enzyme (Boveris and Cadenas, 1997), probably caused by the inflammatory process associated with the venom (França and Málaque, 2003). In this regard, Zamuner et al. (2001) found that Bothrops venoms induce accumulation of leukocytes at the injection site with a local ROS overgeneration including enhanced endogenous production of H$_2$O$_2$. In fact, the increase of H$_2$O$_2$ production in mouse peritoneal leukocytes stimulated with the venom of B. jararaca observed by Zamuner et al. (2001) is related to the increased production of MPO associated with the accumulation of neutrophils in the tissues affected by the venom (Kuebler et al., 1996). Accordingly, Bothrops venom also increased the production of H$_2$O$_2$ in renal proximal tubules of rats (Castro et al., 2003), and CAT rather than GPx activity responds in conditions of large enhancements of endogenous H$_2$O$_2$ production (Halliwell and Gutteridge, 2007).

As found for CAT activity, the profile of GPx activity was very similar, showing significant increases compared to individuals from the control group. Although no significant increases in TBARS levels were detected, GPx is also responsible for H$_2$O$_2$ detoxification and also other hydroperoxides derived from oxidation processes, especially involving lipids (Halliwell and Gutteridge, 2007). This enzyme needs GSH for its function and the increases observed in GPx activity occurred after the snakebite at all times examined, irrespective of the GSH depletion detected after the Bothrops snakebite.
GR allows the continuous conversion of GSSG to GSH, via oxidation of NADPH to NADP (Halliwell and Gutteridge, 2007). As found for the other antioxidant enzymes, blood GR activity in the experimental groups showed no significant differences at the different periods after the Bothrops snakebite, and also when considering individuals from the control group. However, GR activity showed a strong tendency to increase, probably reflecting the depleted levels of GSH found at all times post-snakebite, probably trying to keep high and stable the GSH concentrations of this antioxidant compared to its oxidized (GSSG) form (Halliwell and Gutteridge, 2007).

Both explanations above described could also be applied to the remarkable decrease found in GST activities of snakebite patients at all periods compared to controls. It is important to note that the sharp GST decrease was accompanied by GSH depletion during all the periods examined. GSH is also necessary for GST function, therefore such glutathione paucity could also be responsible for such pronounced decreased activity. In addition, GST regulates the synthesis of leukotrienes and prostaglandins, as well as immune and inflammatory responses, and also participates in the elimination of hydroperoxides, among other products produced endogenously or derived from the metabolism of xenobiotics (Halliwell and Gutteridge, 2007).

GSH depletion might also be involved with increased plasma levels of TNF-α and such relationship has been also found in cultured cells involved with inflammatory processes (Ishii et al., 1992; Phelps et al., 1995). Accordingly, the venom jararhagin, which is present in Bothrops venom, releases TNF-α and interleukins at the snakebite area, as well as in the serum of mice that received injections of this toxin (Clissa et al., 2001). The accumulation of neutrophils in the tissue after injection of jararhagin was described by Costa et al. (2002), which characterize the inflammatory process and oxidative stress inherent to the bothropic snakebite. Curiously, after the snakebite no significant increase in MPO activity was detected, despite that an elevation of plasma cytokines such as TNF-α and IL-6 often occurs, therefore increasing ROS production (Oliveira Jr., 2004). TNF-α also activates inflammatory leukocytes such as macrophages, neutrophils, monocytes and lymphocytes, and induces mononuclear phagocytes and other cells to produce pro-inflammatory cytokines (IL-1, IL-6 and TNF), thus contributing to the accumulation of leukocytes in the inflammatory site (Clissa et al., 2001), thereby increasing local ROS overgeneration.

Probably as a consequence of a systemic ROS overgeneration after Bothrops snakebite, the decrease found in vitamin E plasma concentrations found in all patients could be related to several reasons, including its mobilization, increased catabolism, and its ROS scavenger ability (Halliwell and Gutteridge, 2007). Accordingly, the most important vitamin E antioxidant property is the participation in avoiding propagation reactions along cell membranes and organelles (Mingjian et al., 1992; Sies, 1991; Halliwell and Gutteridge, 2007).
Bothrops safety doses, as those used in the present study, aiming to however it indicates the persistence of the oxidative insult in after the snakebite, no significant changes were found in samples collected at all periods after the Bothrops envenomation. Such unchanged levels of biomarkers of oxidative stress observed in this study could also reflect the depletions of GSH and vitamin E, suggesting that these two important non-enzymatic antioxidants were effective in protecting against the enhancement of lipid and protein damage promoted by the bothropic envenomation.

As a promising perspective, an antioxidant intervention with vitamins E and C might attenuate the persistence of the systemic oxidative stress condition in such patients, as already demonstrated by our group in different chronic diseases, such as in chagasic patients with chronic cardiopathy (Maçao et al., 2007), hepatitis C (Farias et al., 2012), and children with Down Syndrome (Parisotto et al., 2014).

**Conclusions**

In conclusion, the results revealed a marked systemic oxidative stress in victims of Bothrops snakebite compared to controls. Regarding the samples collected in different periods after the snakebite, no significant differences were found, however it indicates the persistence of the oxidative insult in the blood of victims of snakebite up to one month after hospitalization. The use of an antioxidant supplementation in safety doses, as those used in the present study, aiming to attenuate the oxidative insult and its consequences caused by Bothrops envenomation, might be recommended.

**Ethical statement**

To carry out this trial, this protocol was approved by the Ethics in Research of Universidade Federal de Santa Catarina, in April 2009 (protocol 099/09 CEF/UFSC). The experimental protocol met the determining Resolution 196/1996, from the National Health Council, as well as clinical research on ethical, scientific and technical consonance with internationally accepted standards for clinical trials (Standards of Good Clinical Practice). Each patient participating in the project completed the Term of Consent.

**Conflict of interest**

The authors declare that there are no conflicts of interest.

**Acknowledgements**

This work was supported in part by grants from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico/MCT/MSSCTIE-DECIT, proc. 409266/2006-0). DWF is a recipient of a research fellowship from CNPq (proc. 300353/2012-0).

**References**


Ishii, Y., Partridge, C.A., Del Vecchio, P.J., Malik, A.B., 1992. Tumor necrosis factor-α-mediated decrease in glutathione...
increase the sensitivity of pulmonary vascular endothelial cells to H$_2$O$_2$. J. Clin. Invest. 89, 794–802.


