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Original Research Article

Systemic oxidative stress in victims of *Bothrops* snakebites

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ABSTRACT

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 (t_0), 24 hours (t_{24h}), 7 days (t_{7d}) and 30 days (t_{30d}). 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.

Introduction

Ophidic accidents are an important public health problem due to their incidence, morbidity and mortality (Bernarde, 2014). Poisoning caused by snakebites is a major problem in tropical countries due to its high incidence, severity and after-effect, therefore requiring mandatory reporting to the Ministry of Health and also to the health state secretaries (Oliveira et al., 2008; Albuquerque et al., 2013). In Brazil there are around

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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) (Petrecevich et al., 2000; Zamuner et al., 2001). Furthermore, LAAOs contributes to oxidative stress through the generation of hydrogen peroxide (H_2O_2) and superoxide anion (Izidoro et al., 2014) two important precursors of hydroxyl radical ($^{\bullet}OH$) 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 Polydoro Ernani 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 (t_0) and also at 1 (t_{24h}), 7 (t_{7d}) and 30 (t_{30d}) 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 (t_0), at admission in CIT/HU/UFSC; within 24 h (t_{24h}) 16 samples (3 left the hospital before 24 h from the snakebite and did not return); after seven days (t_{7d}) 14 samples (2 additional patients did not return after 1 week from the snakebite); and after 30 days (t_{30d}) 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 H_2O_2 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.

(1963), which represents approximately 95% of non-protein small thiols.

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 375 μl of methanol were added and this mixture was injected in the HPLC device. Isocratical 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}.

Lipoperoxidation (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 H_2O_2 30%, distilled H_2O and NaH_2PO_4 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}.

Chemicals

All the chemicals were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Statistical analysis

The statistical analysis was carried out by analysis of variance (ANOVA) followed by the Tukey–Kramer test. The Student *t*-test was used after the initial determination and the corresponding temporal intervals (days) within each group at the considering the significance level of $\alpha = 0.05$ in both analysis.

Results

Within the first day (t_0, 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} (t_0), 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 (t_0; 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

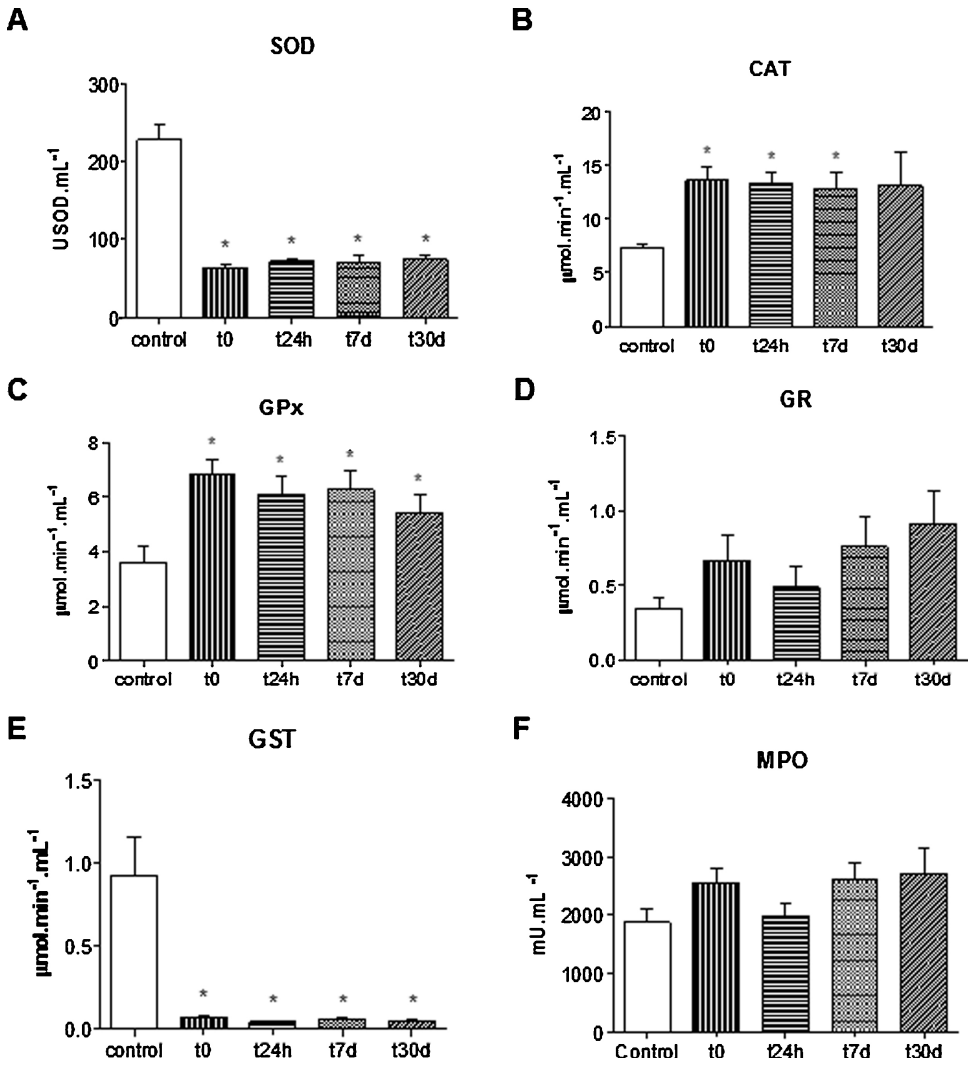

Fig. 1 – Enzymatic activity in hemolysates of (A) superoxide dismutase (SOD), (B) catalase (CAT), (C) glutathione peroxidase (GPx), (D) glutathione reductase (GR), (E) glutathione S-transferase (GST), and of (F) myeloperoxidase (MPO) in serum, in victims of *Bothrops* snakebite. t0: time 0, at admission in the hospital (n = 19); t24h: 24 h after snakebite (n = 16); t7d: seven days after snakebite (n = 14); t30d: 30 days after snakebite (n = 10). *Statistically significant as compared with controls.

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_2O_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_2O_2. In fact, the increase of H_2O_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_2O_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_2O_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_2O_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.

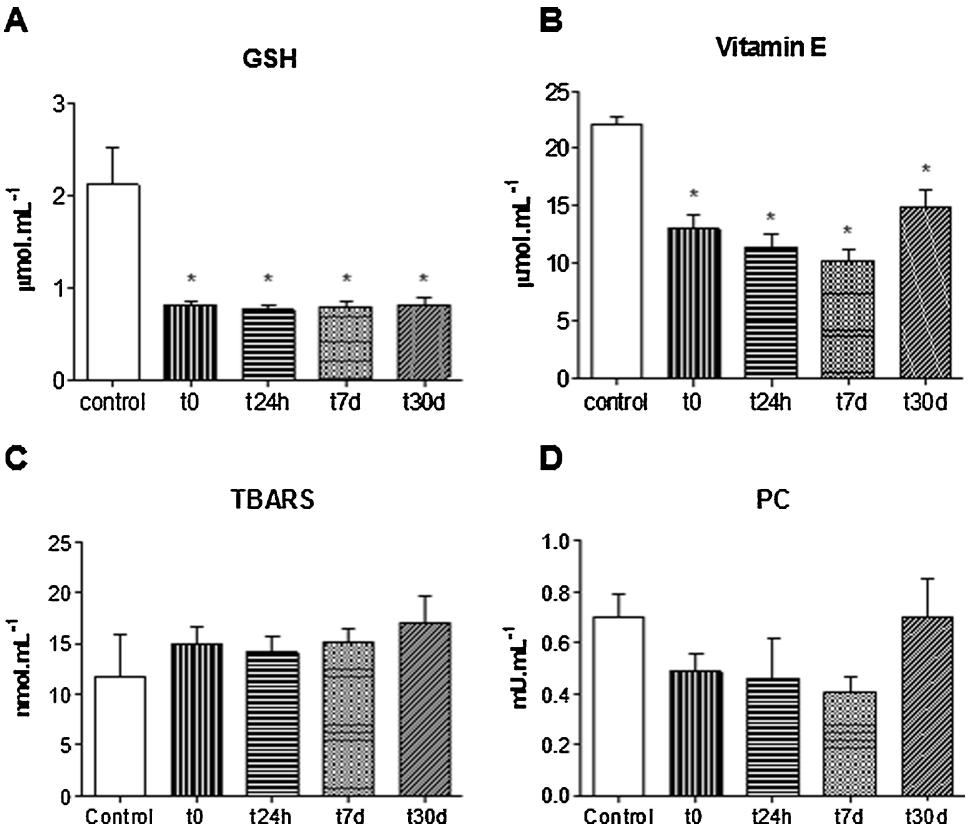

Fig. 2 – Contents of reduced (A) glutathione (GSH) in whole blood extracts, and contents of (B) vitamin E, (C) TBARS (substances that react with thiobarbituric acid), and (D) PC (protein carbonyls) in plasma victims of *Bothrops* snakebite. t0: time 0, at admission in the hospital (*n* = 19); t24h: 24 h after snakebite (*n* = 16); t7d: seven days after snakebite (*n* = 14); t30d: 30 days after snakebite (*n* = 10). Symbols as in Fig. 1.

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). Again, the determinations of vitamin E concentration versus time showed no significant changes, suggesting the persistence of a systemic oxidative stress condition in the blood of snakebite patients.

Despite that higher levels of lipid peroxidation are usually associated with inflammatory processes (Yadav et al., 2002) no significant changes in TBARS and PC concentrations were found in samples collected at all periods after the *Bothrops* snakebite. 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 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 CEP/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.

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