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Serum proteins and electrophoretic profile in horses undergoing crotalid venom hyperimmunization

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Indisputably, the use of antivenoms for the treatment of snakebite envenoming is beneficial for the victims. However, there are few studies addressing the effect of long-term hyperimmunization in inoculated horses. It is known that the injection of snake venoms and adjuvants leads to local and systemic reactions in horses, but little is known about the response of inflammatory proteins. The aim of this study was to evaluate serum proteins and the electrophoretic profile of horses undergoing crotalid venom hyperimmunization. Twenty horses were divided into two groups: an inoculated group, comprising ten horses that were already being used for production of a Crotalus sp. antivenom, and a control group, comprising ten animals that had never been used for hyperimmunization. All animals were clinically healthy and without laboratory abnormalities. Total protein and albumin concentrations were measured in serum. Serum globulins were obtained by calculation. Plasma fibrinogen estimates were determined by the heat precipitation method. Serum proteinograms were obtained using agarose gel electrophoresis. The results revealed a significant increase in the concentrations of total serum proteins, globulins, and β-globulins in the inoculated group, exceeding the reference values. There were slight increases in the α-1- and α-2-globulin subfractions in serum-producing horses, with no statistical significance. We also observed that horses used to produce hyperimmune plasma developed hypoalbuminemia, although the decrease in albumin production was not statistically significant. Our findings suggest that the continuous use of horses to produce crotalid antivenom may lead to a chronic inflammatory stimulus, with changes in plasma levels of inflammatory proteins.

Key words: *hyperimmune plasma, inflammation, proteinogram*

Accidents involving venomous animals, particularly snakebite envenomation, are included by the World Health Organization in the list of Neglected Tropical Diseases which often affect poor populations [21]. These occurrences are a public health problem, not only because of their frequency but also because of the possible severity of envenoming outcomes and sequelae [16, 22].

The ophidian fauna of Brazil comprises about 10% of all known snake species in the world and is divided into ten families [5, 16]. The species Crotalus durissus (Viperidae: Crotalinae) is endemic to the Cerrado and Caatinga Brazilian biomes and easily recognized by the presence of corneous rings in the tail. On the other hand, the distribution of Crotalus durissus has expanded to areas of the Atlantic Rainforest due to high deforestation rates, agricultural impacts, the presence of livestock, industrialization, and disordered urbanization [10].

The use of antivenom as a specific treatment for snake-

bites is recognized by the Brazilian Ministry of Health as beneficial and effective for victims [3]. Scientific and technological research are enabling improvements in the quality of antivenoms; however, there are few studies regarding the pathophysiological changes in hyperimmune plasma produced in horses. This is of special importance since sudden death without any previous clinical signs is likely in the animals used for this purpose [17].

Inflammation is a nonspecific response of the host that occurs after a tissue injury. Adjuvant-induced inflammation is a known reality in veterinary medicine [13]. After the inoculation of snake venoms and adjuvants, different reactions have been noticed in horses, including swelling, lumps, abscesses, fistulas, and fibrosis at the site of injection [1, 20]. In addition to local reactions, signs of chronic inflammatory response have also been reported in horses subjected to repeated complete Freund's adjuvant administrations [13].

Serum proteins constitute a large group of hundreds of different proteins with varied functions and structures [19]. Based on the principles of electrophoresis, serum proteins are divided into albumin and α-, β-, and γ-globulins. Among the α- and β-globulins, there are proteins such as fibrinogen and acute phase proteins (APPs) as well as components of the complement system and coagulation factors [19]. APPs are used to access the innate immune system for a systemic response to infection, inflammation, or trauma [14, 15].

Slight changes in haematological and serum biochemical parameters in horses during snake antivenom production have been described [20]. However, to the best of our knowledge, there is still a need for studies addressing the electrophoretic profile of inflammatory proteins in these animals.

Considering that hyperimmunization programs may elicit an inflammatory response, the aim of this study was to evaluate the serum proteins and electrophoretic profile of horses used for the production of crotalid antivenom.

Materials and Methods

This study was performed according to the Ethical Principles for Animal Experimentation determined by National Brazilian Law 11.794/08, with the approval of the Committee on Ethics in the Use of Animals (CEUA/UFF number 292) and authorization by the Instituto Vital Brazil.

Animals and protocol for immunization

Twenty mixed-breed horses from the Instituto Vital Brazil Farm (IVB Farm), weighing approximately 350 kg, were divided into two groups of ten horses each: a) four females and six males from 7 to 17 years of age that were being used for the production of *Crotalus durissus terrificus*

antivenom (inoculated group) and b) seven females and three males from 4 to 11 years of age that had never been used for the production of hyperimmune plasma (control group).

All animals were clinically healthy on physical examination and without laboratory abnormalities in their complete blood counts and biochemistry profiles [9]. They were subjected to prophylactic vaccination and deworming programs that were already part of the IVB farm routine, in which they were also tested and periodically monitored for equine infectious anaemia and glanders (*Burkholderia mallei*) in accordance with Brazilian Ministry of Agriculture regulations.

Each horse in the inoculated group was subcutaneously prime immunized with 5 mg of *Crotalus durissus terrificus* crude venom, administered eight times at seven-day intervals. These animals also received six re-immunizations with 4 mg of *Crotalus durissus terrificus* crude venom administered annually at two-month intervals. The average time these animals spent in the inoculation program was 6.3 ± 1.3 years.

Serum proteinogram, fibrinogen, and protein electrophoresis

Jugular blood samples were collected in EDTA and anticoagulant-free tubes two weeks after the bloodletting stage of the immunization protocol in order to avoid the acute effect of hyperimmunization on protein concentrations. The samples were kept under refrigeration and immediately transported to the laboratory for processing. The anticoagulant-free samples were centrifuged to harvest serum, which was then stored at −20°C in sterile microtubes to be analysed later.

Serum was analysed in a semi-automatic spectrophotometer (Bio200, Bioplus Ltda, Barueri, Brazil) using commercial kits (Labtest Diagnóstica S.A., Lagoa Santa, Brazil) according to the manufacturer's recommendations. Measurement of total serum proteins was carried out by the biuret reaction. Albumin was measured by the colorimetric technique, using bromocresol green dye. The globulin fraction was obtained by subtracting albumin from the total serum proteins. Plasma fibrinogen determination was performed by the heat precipitation method, using whole blood from EDTA tubes [7].

To detect changes in the profile of serum proteins, agarose gel electrophoresis was performed with agarose films (CELM, Barueri, SP, Brazil) and an UP-250 electrophoresis system (CELM, Barueri, Brazil). After protein separation at pH 9.5 in Tris buffer, the gels were stained with Amido Black 0.2% in 5% acetic acid solution and then scanned and analysed with a flatbed scanner using the SDS-60 software.

Statistical analysis

Descriptive analyses were performed for the serum proteinogram, fibrinogen, and protein electrophoresis concentrations of each group. The Kolmogorov-Smirnov test (KST) was performed for each variable result for choosing subsequent analysis. The independent samples t-test was used to compare results between the inoculated and control groups, and the level of significance was set at 5%. All analyses were performed using the IBM SPSS Statistics v.21 software.

Results

Data for total serum proteins, fractions, protein electrophoresis, and fibrinogen are shown in Table 1. There were increases (*P*<0.05) in the concentrations of total proteins and globulins in the hyperimmune plasma-producing horses which even exceeded the normal ranges. Regarding the serum protein electrophoresis results, the β-globulin level in the inoculated group showed a significant increase (*P*<0.05), also exceeding the reference value.

Discussion

Previous studies have already sought to relate the presence of clinical, haematological, and biochemical abnormalities with the use of hyperimmune plasma-producing horses; however, the changes observed were mostly slight and transient [1, 20].

The acute inflammatory response is a complex reaction that begins in response to tissue damage resulting from an inflammatory, infectious, immunological, neoplastic, or traumatic cause [4]. The injury leads to the release of inflam-

matory cytokines, which trigger the production of positive APPs [12]. Thus, in response to an inflammatory stimulus, positive APPs initially increase in the serum component, remain elevated while the pro-inflammatory stimulus is taking place, and drop in response to treatment, assuming diagnostic and prognostic roles [11].

In the present study, a significant increase in the total serum protein concentration was observed, resulting in hyperproteinemia in the hyperimmunized horses compared with the horses in the control group. Increases in serum proteins were also reported in horses undergoing immunization to produce polyvalent snake antivenom [1, 20], particularly in their β-globulin values [20]. Our results also showed a statistically significant increase in the serum globulin concentrations and β-globulin range of the inoculated group, exceeding the normal values for this species.

C-reactive protein, which is produced by the liver, is considered a positive APP found in the β-globulin fraction. The serum levels of C-reactive protein rise dramatically after stimulation of inflammatory cytokines [8]. In this context, it is possible that the increase in β-globulins observed here occurred due to an inflammatory stimulus in the hyperimmunized horses.

Another aspect to be considered in the electrophoretic profile of the serum proteinogram was the lack of statistical significance in the mean values of the α-globulins. The α-globulins include several proteins, including haptoglobin and ceruloplasmin, and are classified as positive APPs because their serum concentrations are increased during inflammatory processes [18]. However, this correlation was not evident in our results. On the other hand, chronic inflammation results from a set of ongoing inflammatory stimuli, so the increase in APP concentration may be quite

Table 1. Mean ± standard deviation and *P*-values for the serum proteinogram, fibrinogen and protein electrophoresis of horses never used in immunization programs (control group) and horses used for the production of anticrotalic hyperimmune plasma (inoculated group).

Parameter	Reference value[1]	Control group (n=10)	Inoculated group (n=10)	*P*-value
		Mean[2] ± SD		
Serum proteinogram				
Total protein (g/d*l*)	5.2–7.9	6.65[A] ± 0.53	8.20[B] ±0.56	0.000
Albumin (g/d*l*)	2.6–3.7	2.43[A] ± 0.32	2.18[A] ± 0.52	0.215
Globulins (g/d*l*)	2.62–4.04	4.22[A] ± 0.45	6.02[B] ± 1.03	0.000
Protein electrophoresis				
Albumin (g/d*l*)	2.6–3.7	2.66[A] ± 0.55	2.17[A] ± 0.44	0.410
Alpha 1 globulin (g/d*l*)	0.06–0.7	0.29[A] ± 0.14	0.37[A] ± 0.12	0.147
Alpha 2 globulin (g/d*l*)	0.3–1.3	0.37[A] ± 0.12	0.59[A] ± 0.22	0.130
Beta globulin (g/d*l*)	0.7–2.5	2.16[A] ± 0.37	2.99[B] ± 0.57	0.001
Gama globulin (g/d*l*)	0.55–1.9	1.53[A] ± 0.37	1.58[A] ± 0.32	0.756
Fibrinogen (mg/d*l*)	100–400	380.00[A] ± 147.57	300.00[A] ± 141.42	0.232

[1]According to Kaneko, Harvey and Bruss (2008) [9]. [2]Different letters (A/B) in the same line indicate a statistically significant difference (*P*<0.05) by the independent samples *t*-test. SD: standard deviation.

inferior to that observed after a single acute event of inflammation [8]. This could explain why we did not observe a significant increase in the α-globulin subfractions of our inoculated horses. It is noteworthy that we did notice a slight but non-significant increase in the α-1- and α-2-globulin subfractions when comparing the control and inoculated groups.

A drop in serum albumin values was previously reported [20]. Our results also showed a small but non-significant decrease in the serum albumin concentration of the hyperimmunized horses, dropping it to below the normal limit. Despite not being statistically significant, we cannot refute the fact that this behaviour is compatible with the profile of albumin, which is considered a negative APP because its serum concentration decreases during the inflammatory response [4, 8, 19].

Plasma fibrinogen is well accepted for the detection of inflammatory diseases in horses, even though it is considered a slow-reacting protein [6, 23]. Unexpectedly, there was no significant difference in the plasma fibrinogen concentration between the two groups evaluated in the present study. This result conflicts with the previously published data [13], in which plasma fibrinogen concentrations increased after the first two injections of adjuvant and remained elevated throughout the study. On the other hand, measurement of other serum parameters of horses provides better evidence of inflammation than the fibrinogen concentration [2].

The lack of an increase in the gamma-globulin fraction was expected, since the blood samples were taken when the hypergammaglobulinemia caused by hyperimmunization was no longer present, reflecting the basal state of the animals used in the inoculated group.

The decline in serum concentrations of APPs in patients coincides with their recovery or treatment and is generally regulated by feedback mechanisms that limit the inflammatory response and lead to its resolution if no further stimulus occurs [8]. However, when animals receive repeated and consecutive inflammatory pulses, the inflammatory response can become chronic, and under these conditions, a prolonged increase in APPs is generally observed, although the serum concentrations in these cases are not as high as after a single event of inflammation.

The results of this study suggest that the inoculum dose of the toxin, the inoculation interval to produce antivenom, or a combination of the two causes a chronic inflammatory condition characterized by changes in the proteinogram, mainly in beta-globulin fraction. Despite these findings having great potential for future use to promote animal welfare in horses kept in crotalid antivenom production programs, the design of our study was insufficient to determine which would be the best protocol.

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