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Hematological dynamics of horses producing antibothropic serum during the immunization process

[*Dinâmica hematológica de cavalos produtores de soro antibotrópico, durante o processo de imunização*]

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ABSTRACT

Ophidic accidents caused by snakes of the genus Bothrops are responsible for 70% of notifications in the country. The antiophidic serum is the specific antidote against envenomation caused by these snakes. Aiming to evaluate the health status of horses producing antibotropic serum, the hematological variables and serum iron were delineated along the protocol of three immunizations. The animals were evaluated at 17 experimental moments, being: control (before the immunogen application), four hours, 24h, 48h, 72h and seven days after inoculations, for each immunization. There was a significant decrease in erythrocyte, hemoglobin, and hematocrit values after the second immunization. Serum iron values showed significant decreases during the three immunizations. Significant decreases in lymphocyte values were noted in the first two immunizations, while there was a significant increase in neutrophil values during all three immunizations. Eosinophils, monocytes, and basophils did not show significant difference throughout the three immunizations. The immunization protocol described in this study caused an inflammatory reaction at the site of immunogen application and transient changes in hematological parameters and iron levels, as by the end of the immunization protocol, the variable values returned the reference levels.

Keywords: equine, inflammation, serum iron, *Bothrops* spp

RESUMO

Acidentes ofídicos causados por serpentes do gênero Bothrops *são responsáveis por 70% das notificações no país. O soro antiofídico é o antídoto específico contra o envenenamento causado por essas serpentes. O objetivo do presente estudo foi avaliar o estado de saúde de equinos produtores de soro antibotrópico, pelo monitoramento dos parâmetros hematológicos e do ferro sérico, durante a realização do protocolo de três imunizações. Os animais foram avaliados em 17 momentos experimentais: controle (antes da aplicação do imunógeno), quatro horas, 24h, 48h, 72h e sete dias após as inoculações, para cada imunização. Houve diminuição significativa nos valores dos eritrócitos, da hemoglobina e do hematócrito após a segunda imunização. Verificaram-se diminuições significativas nos valores do ferro durante as três imunizações. Foram verificadas diminuições significativas nos valores dos linfócitos, nas duas primeiras imunizações, e aumento significativo dos valores dos neutrófilos nas três imunizações. Os eosinófilos, monócitos e basófilos não apresentaram diferença significativa ao longo das três imunizações. O protocolo de imunização descrito neste trabalho causou reação inflamatória no local da aplicação do imunógeno e reações transitórias dos parâmetros hematológicos e do ferro, pois, no final do protocolo de imunização, os valores das variáveis retornaram aos intervalos de referência.*

Palavras-chave: equinos, inflamação, ferro sérico, Bothrops *spp*

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INTRODUCTION

Antivenom is the recommended effective treatment for envenomation incidents with venomous snakes and should be administered promptly (Ministério da Saúde, 2021; Guidelines…, 2018). In 2007, the World Health Organization included antivenom in the Model List of Essential Medicines for Basic Health Care (Guidelines…, 2018). Monthly, the Ministry of Health distributes antivenom quotas to the states, considering the data collected by SINAN, the Notifiable Diseases Information System (Ministério da Saúde, 2019).

The manufacturing of antivenom relies on animals at every stage of the processing, whether it is the venom extraction from snakes, the production of hyperimmune serum by horses, safety, titration and potency tests conducted on mice and guinea pigs, as well as pyrogen testing on the serum and the final product, consequently the World Health Organization recommends standards to minimize discomfort and pain and to ensure the welfare os these animals (Guidelines…, 2018).

Horses are domesticated, large animals (which allows for the collection of a significant volume of blood at once) and are easy to handle, making them the preferred choice for antivenom production (Guidelines…, 2018). Although horses have been used for over a century in antivenom production (Soros…, 2018), studies monitoring the effects of this production on horses have been conducted by Castillho *et al*. (2022) and Huerta *et al*. (2023).

Over 130 million years of snake evolution, the bioactive elements of their venom have become more potent (Araújo and Souza, 2007), reaching a level where snake venoms are possibly the most complete of all venoms (Cardoso *et al*., 2009).

The venoms are a mixture of organic and inorganic compounds, with 90% to 95% of the dry weight composed of protein (Araújo and Souza, 2007; Cardoso *et al*., 2009). These proteins, called proteases, have enzymatic activity on many substrates (Araújo and Souza, 2007). The main representatives of this group for the genus *Bothrops* (jararaca) are metalloproteases and serine proteases (Araújo and Souza, 2007; Cunha and Martins, 2012), which lead to the interruption of homeostasis, with serious damage to tissues near or far from the site of the bite (Araújo and Souza, 2007). Metalloproteases, for example, are related to local inflammatory reactions (Zychar *et al*., 2021). Phospholipases, disintegrins, myotoxins, and neurotoxins are also part of this group (Cunha and Martins, 2012). These, together with the other components of venom, are capable of triggering different pathological processes in living beings (Araújo and Souza, 2007).

Bothropic venom has three pathophysiological activities: proteolytic (responsible for the acute inflammatory response), coagulant and hemorrhagic (Cardoso *et al*., 2009). Proteolysis, inflammation, and subsequent necrosis are caused by proteolytic enzymes, vasoactive substances such as bradykinin and histamine, resulting in intense local inflammation with edema, pain, and congestion (Barraviera and Pereira, 1991; Kouyoumdjian *et al.,* 1990; Fonteque *et al.,* 2001). The coagulant action is a result of the venom's ability to activate coagulation factors such as fibrinogen, prothrombin, and factor X. Hemorrhagic activity is attributed to specific components known as hemorrhagins, zinc-containing metalloproteases that can disrupt the integrity of the vascular endothelium and possess disintegrin activity. Additionally, they are platelet aggregation inhibitors (Kouyoumdjian, *et al.,* 1990; Cardoso *et al.,* 2009).

Studies related to bothropic accidents in animals are scarce, in contrast to crotalic accidents (Lopes, *et al.,* 2012; Sampaio *et al.,* 2018).

The objectives of this study were to evaluate and monitor the health status of horses producing antibothropic serum during the immunization period, with a focus on monitoring hematological parameters and serum iron concentration.

MATERIALS AND METHODS

The project was approved by the Ethics Committee for Animal Use (CEUA) of the Agricultural Sciences Department at the Federal University of Paraná (number 009/2020).

In this study, 15 clinically healthy horses (four females and 11 males) of undefined breed aged

between seven and 21 years, with an average weight of 496.7±51.9kg were used.

These animals belong to the Center for the Production and Research of Immunobiologicals (CPPI) located in Piraquara, Paraná. They are kept in Tifton grass pasture (*Cynodon dactylon*), with access to water *ad libitum*, and receive two kilograms of maintenance feed (PróEquino Agrária Nutrição Animal - Guarapuava, PR, Brazil), containing 14% protein and 3% ether extract, mixed with 25g of mineralized salt, two kilograms of alfalfa hay, and half a kilogram of germinated oats, twice a day.

The animals were vaccinated against influenza, rhinopneumonitis, encephalomyelitis, leptospirosis, *Streptococcus equi*, tetanus, and rabies. Antiparasitic control was carried out periodically.

Prior to the immunization protocol, the horses were kept inactive for 60 days, meaning they were allowed to graze in the Tifton pasture and received the same diet to recover.

Before being included in the research, the animals underwent a clinical examination (Feitosa, 2020). Inclusion criteria encompassed normal parameters for heart rate, respiratory rate, and temperature, as well as hematological parameters and biochemical profiles (plasma proteins, fibrinogen, urea, creatinine, creatine kinase, aspartate aminotransferase, and gamma glutamyltransferase) which presented results within the reference range for equines (Kaneko *et al*., 2008).

The venom was produced in the Venom Production Section at CPPI. Its concentration is 1 mg/mL: a mixture of 5mL of 0.85% saline solution and 5mg of the "bothropic pool" (50% *Bothrops jararaca*, 12.5% *Bothrops jaracussu*, 12.5% *Bothrops neuwiedi*, 12.5% *Bothrops moojeni*, 12.5% *Bothrops alternatus*).

The first immunization was carried out using a preparation of 5mL of Freund's incomplete adjuvant plus 5 mL of the bothropic solution (5mg of the "bothropic pool" and 5mL of 0.85% saline solution). Fourteen days after the first immunization, the second immunization was performed with 5mL of 4% aluminum hydroxide plus 5 mL of the bothropic solution. Seven days after the second immunization, the third one was performed with 5mL of 4% aluminum hydroxide plus 5mL of the bothropic solution, following the CPPI protocol.

After shaving and local antisepsis, 10.0mL of the immunogen were administered subcutaneously in the dorsal region at five distinct and equidistant points, with 2.0mL per point. The immunization protocol was carried out in the same manner for all animals.

Blood samples were collected at 17 different time points, both before and during the immunization process, through jugular vein puncture. The samples were placed in tubes without anticoagulant (BD Vacutainer® Blood Collection Tube; Becton, Dickinson and Company, USA) and in tubes with the anticoagulant ethylenediaminetetraacetic acid, EDTA (BD Vacutainer® K2 EDTA Blood Collection Tube; Becton, Dickinson and Company, USA), after local antisepsis.

A total of 4mL of whole blood and 8mL of blood for serum separation were collected. The blood collections were conducted between 8:00 and 10:00 in the morning. Blood samples were collected before venom application (control), at 4 hours, 24 hours, 48 hours, 72 hours, and seven days after venom application.

The first immunization ("bothropic pool" plus Freund's incomplete adjuvant) occurred on day zero; the second ("bothropic pool" plus 4% aluminum hydroxide), 14 days after the first; and the third ("bothropic pool" plus 4% aluminum hydroxide), 21 days after the first immunization. Blood sample collection times were the same for all three immunizations: first immunization (day 0): M0 (control), M1 (4h), M2 (24h), M3 (48h), M4 (72h), M5 (seven days). Second immunization (day 14): M6 (M0 of the 2nd immunization), M7 (4h), M8 (24h), M9 (48h), M10 (72h), M11 (seven days). Third immunization (day 21): M11 (M0 of the 3rd immunization), M12 (4h), M13 (24h), M14 (48h), M15 (72h), M16 (seven days). The time points seven days after the second immunization and the control for the third immunization coincide and were designated as M11. At this time (M11), blood collection was conducted before venom application.

The samples were centrifuged (Fanem Centrifuge, Excelsa II 206 – BL model, São Paulo, Brazil) for 10 minutes at 1,500 rotations per minute to obtain serum. Serum aliquots were stored in 2.0mL Eppendorf tubes and frozen at -80°C until the time of analysis.

Iron concentrations were determined using the modified Goodwin method with a commercial kit (Bioclin® - Quibasa Química Básica Ltda, Belo Horizonte, MG, Brazil) in an automatic biochemical analyzer (BS-200® Mindray automatic chemistry analyzer, Shenzen, China) at the Veterinary Clinical Pathology Laboratory of the Federal University of Paraná (UFPR).

Hematological parameters were determined in whole blood samples. Total erythrocyte and leukocyte counts and hemoglobin concentration were determined using the Mindray® BC 2800 VET model. Hematocrit was carried out using the microhematocrit technique (Jain, 1986). Hematimetric indices (mean corpuscular volume – MCV and mean corpuscular hemoglobin concentration – MCHC) were calculated (Wintrobe, 1932). Leukocyte differential counts were performed on blood smears stained with May-Grünwald Giemsa, immediately after sample collection.

Statistical analyses were carried out using the *SPSS* (Statistical Package for the Social Science) version 20, *GraphPad Prism* 10.0.2 (232), and *Statistica Single User* version 13.2 software. Due to the non-normality of data in most time points, as determined by the *Shapiro-Wilk* test ($p<0.05$), non-parametric tests were chosen for analysis. The paired Friedman ANOVA test was used for comparisons between the evaluated time points, followed by the Dunn test when $p<0.05$ (indicating a difference between the evaluated time points). The level of significance adopted for the tests was 5%, meaning that comparisons with $p<0.05$ were considered significant. The results are presented in the form of graphs.

RESULTS

A reaction at the site of immunization was observed in all horses, including an increase in temperature, edema, abscesses, and fistulas, regardless of the adjuvant used.

At the time point immediately before the 1st immunization (M0), the investigated blood variables presented results within the reference ranges for horses, as follows: serum iron concentrations: 73.14 – 140.14µg/dL (Kaneko *et al.*, 2008); number of erythrocytes: 6.4 – 10.0 x 106/µL; total leukocyte count: 5,200 – 13,900/µL; segmented neutrophil count: 2,200 – 7,400/µL; lymphocyte count: 1,100 – 5,300/µL; eosinophil count: 0 - 600/µL; monocyte count: 0 – 900 /µL; basophil count: 0 - 300/µL (Jain, 1986; Weiss and Wardrop, 2010).

A significant decrease in serum iron concentration ($P < 0.05$) was observed after the 1st immunization at 24 hours (M2), 48 hours (M3), 72 hours (M4), and seven days (M5). After the 2nd immunization (M6), there was a significant decrease ($P < 0.05$) at 24 hours (M8), 48 hours (M9), and 72 hours (M10). Following the 3rd immunization (M11) there was a significant decrease ($P < 0.05$) at 24 hours (M13) and 48 hours (M14) (Figure 1).

The results of hematological parameters are presented in Figures 2 to 7. There was a significant decrease in the number of erythrocytes ($P < 0.05$) only after the 2nd immunization at 24 hours (M8) and 72 hours (M10) (Figure 2). Similarly, a significant decrease in hematocrit values ($P < 0.05$) was observed after the 2nd immunization at 72 hours (M10) (Figure 3). There was a significant decrease in hemoglobin concentrations after the 1st immunization at 72 hours (M4) and after the 2nd immunization at 72 hours (M10) (Figure 4).

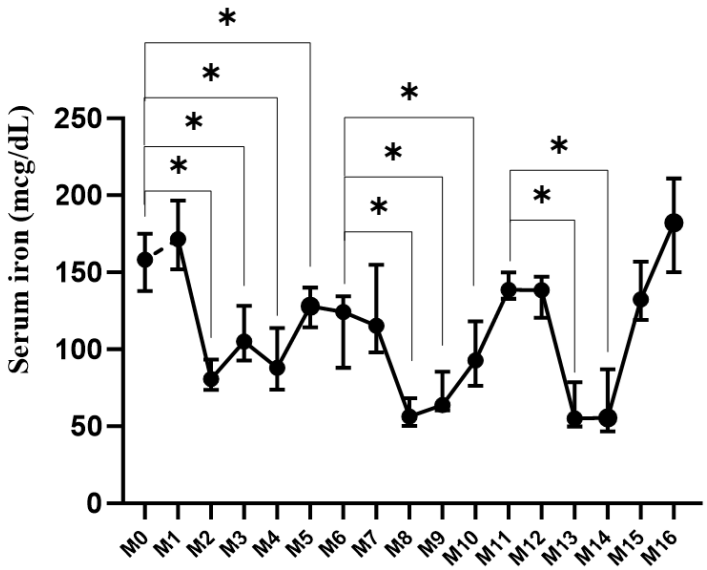

Figure 1. Distribution, at 17 distinct time points, of the serum iron concentration (µg/dL) of 15 horses submitted to three immunizations with venom from snakes of the genus *Bothrops* spp. Circles represent medians, and error bars depict the interquartile range. Asterisks indicate significant differences between time points in comparison with each immunization (M0; M6; M11): *p <0.05.

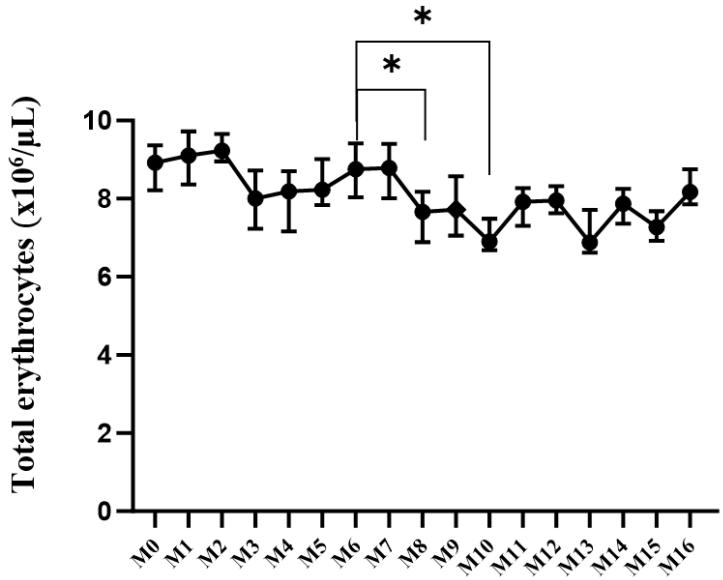

Figure 2. Distribution, at 17 distinct time points, of the erythrocyte concentration (x106/µL) of 15 horses submitted to three immunizations with venom from snakes of the genus *Bothrops* spp. Circles represent medians, and error bars depict the interquartile range. Asterisks indicate significant differences between time points in comparison with each immunization (M0; M6; M11): *p <0.05.

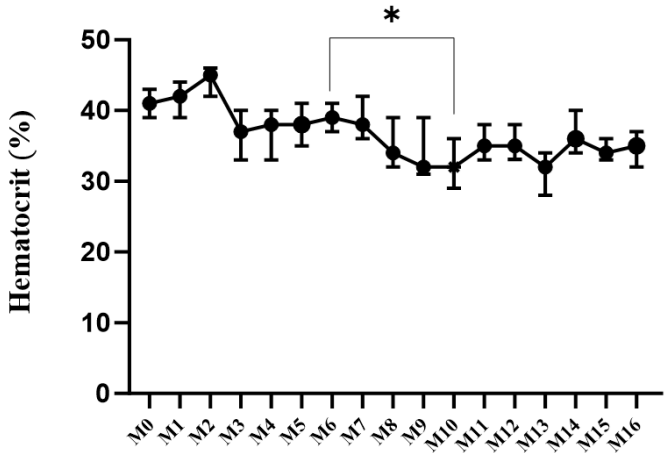

Figure 3. Distribution, at 17 distinct time points, of the hematocrit (%) of 15 horses submitted to three immunizations with venom from snakes of the genus *Bothrops* spp. Circles represent medians, and error bars depict the interquartile range. Asterisks indicate significant differences between time points in comparison with each immunization (M0; M6; M11): *p <0.05.

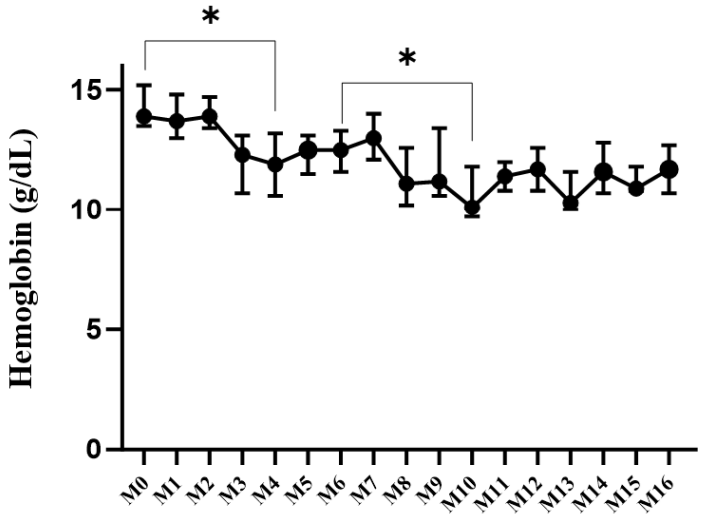

Figure 4. Distribution, at 17 distinct time points, of the hemoglobin concentration (g/dL) of 15 horses submitted to three immunizations with venom from snakes of the genus *Bothrops* spp. Circles represent medians, and error bars depict the interquartile range. Asterisks indicate significant differences between time points in comparison with each immunization (M0; M6; M11): *p <0.05.

The median values of MCV ranged from 43.7 to 48.6 fL during the immunization protocol. There was a significant decrease ($P < 0.05$) after all three immunizations, including at 4 hours (M1), 24 hours (M2), 48 hours (M3), 72 hours (M4), and seven days (M5) after the 1st immunization, at 48 hours (M9) and 72 hours (M10) after the 2nd immunization, and at 48 hours (M14) after the 3rd immunization. The median values of MCHC ranged from 31.9 to 34.4% throughout the immunizations. There was a significant decrease ($P < 0.05$) at M1 and M11 and a significant increase ($P < 0.05$) at M7, M10, and from M13 to M15.

There was a significant increase in the total numbers of leukocytes (Figure 5) and segmented neutrophils (Figure 8) ($P < 0.05$) after all three

immunizations, including at 24 hours (M2), 48 hours (M3), and 72 hours (M4) after the 1st immunization, at 24 hours (M8) and 48 hours (M9) after the 2nd immunization, and at 24 hours (M13) and 48 hours (M14) after the 3rd immunization. There was a significant decrease ($P < 0.05$) in lymphocyte concentration (Figure 9) after the 1st immunization at 48 hours (M3) and at 24 hours (M8) after the 2nd immunization. No significant differences were observed in eosinophil, monocyte, and basophil concentrations between the evaluated time points during the three immunizations (results not shown).

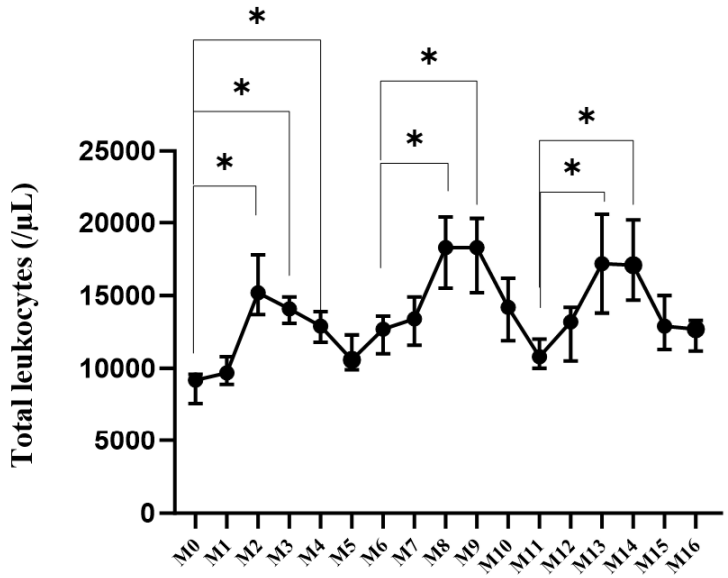

Figure 5. Distribution, at 17 distinct time points, of the total white blood cell count (/µL) of 15 horses submitted to three immunizations with venom from snakes of the genus *Bothrops* spp. Circles represent medians, and error bars depict the interquartile range. Asterisks indicate significant differences between time points in comparison with each immunization (M0; M6; M11): *p <0.05.

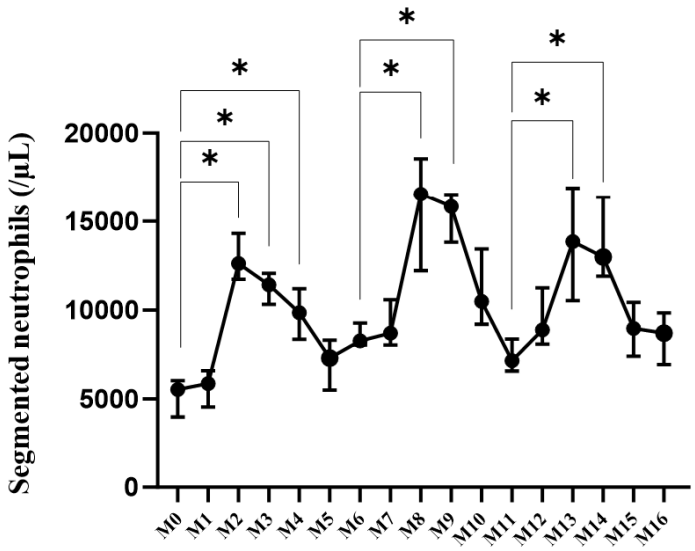

Figure 6. Distribution, at 17 distinct time points, of the segmented neutrophil count (/µL) of 15 horses submitted to three immunizations with venom from snakes of the genus *Bothrops* spp. Circles represent medians, and error bars depict the interquartile range. Asterisks indicate significant differences between time points in comparison with each immunization (M0; M6; M11): *p <0.05.

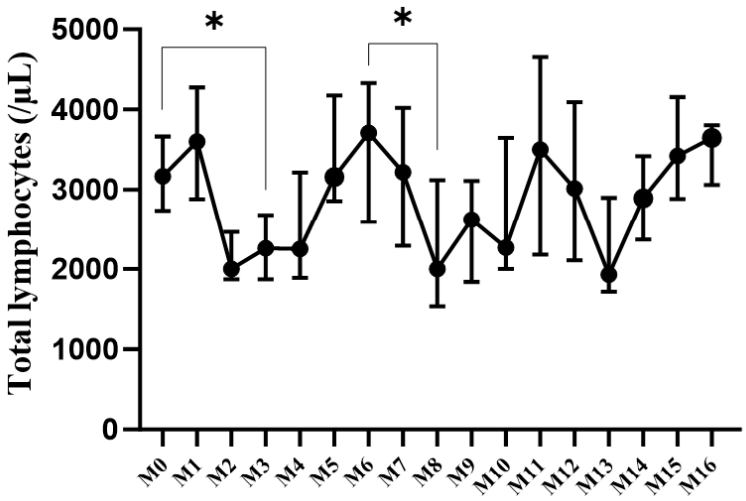

Figure 7. Distribution, at 17 distinct time points, of the lymphocyte count (/µL) of 15 horses submitted to three immunizations with venom from snakes of the genus *Bothrops* spp. Circles represent medians, and error bars depict the interquartile range. Asterisks indicate significant differences between time points in comparison with each immunization (M0; M6; M11): *p <0.05.

DISCUSSION

One of the most important objectives in the production of hyperimmune serums is to find a balance between the safety of immunized horses and the effectiveness of the product (Arguedas *et al*., 2022).

Literature shows that both snake venom and the adjuvant used in immunization can cause local reactions at the injection site (Huertas *et al*., 2023; Arguedas *et al*., 2022; Araújo and Belluomini, 1960). Inoculating the same amount of venom with different adjuvants caused reactions at the immunization sites in horses, with more severe local lesions promoted by Freund and Montanide adjuvants, which also promoted a greater immune response and a higher ability to neutralize lethal venoms (Arguedas *et al*., 2022). These adjuvants promote immunostimulation, facilitating the response to antigens. Therefore, they are important coadjuvants in the production of hyperimmune serums and vaccines (Hanly *et al*., 2003; Spinosa *et al*., 2011). In the antivenom serum-producing horses in this study, local inflammatory reactions (abscesses, edema, and fistulas), in addition to the venom itself, may have been caused by the use of Freund's incomplete adjuvant in the first immunization and 4% aluminum hydroxide in the second and third immunizations.

In a study by Araújo and Belluomini (1960), horses injected with 1 mg of bothropic venom (a mixture of different *Bothrops* species) per kilogram of body weight in the dorsal region via intramuscular injection developed reactions at the site of venom application, including bleeding and edema. Local reactions caused by bothropic venom result from the action of proteases, inflammatory mediators, and various other enzymes. The metalloproteinases present in jararaca venom cause inflammatory reactions at the bite site, as they equivalently modulate adhesion molecules, which in turn recruit leukocytes (Zychar *et al*., 2021).

The 15 horses of this study did not present anemia, but rather a decrease in erythrocyte values during the immunization process, and concomitant neutrophilia. According to Weiss *et al*. (2019), anemia may occur due to inflammation because iron homeostasis is influenced by pro-inflammatory cytokines, which increase the production of hepcidin by the liver. Hepcidin, in turn, inhibits the action of ferroportin, resulting in iron retention within reticuloendothelial cells, leading to erythropoiesis with iron restriction. Additionally, there is synthesis and sequestration of apoferritin within macrophages, resulting in hypoferremia, which starts at 24 hours into the early stages of inflammation, and is only normalized when the

inflammatory insult is resolved (Schliewert *et al*., 2022; Harvey, 2012).

The decrease in erythrocyte parameters observed in the 15 horses can be explained by the fact that the production of antibothropic serum in horses promotes local inflammatory reactions and a systemic mechanism of immunological adaptation. As a result, erythrocytes with opsonization of IgG on their membranes are removed by the mononuclear phagocytic system, an effect amplified by complement system signaling. This leads to the phagocytosis of these cells and the sequestration of iron within macrophages, reducing the duration of erythrocytes in the bloodstream, which is approximately 140 to 145 days in horses (Christian, 2010). Any changes in circulating erythrocytes allow us to inquire about the distribution of iron in the body (Cook *et al*., 1992). Despite significant variations in MCV and MCHC values, there were no morphological changes in erythrocytes as the medians of these indices were within the reference ranges for the species (Thomassian 1996).

Therefore, the decrease, after the three cycles of bothropic venom inoculation, in the values of variables (approaching the lower limit of the reference range for the species) such as erythrocyte, hemoglobin, hematocrit, and persistent hypoferremia suggest indirect inhibition of erythropoiesis and an increase in the removal of red blood cells through immune-mediated damage (Poitout-Belissent and McCartney, 2010). This may occur during the immunization protocols. Additionally, there is synthesis and sequestration of apoferritin within macrophages, resulting in hypoferremia, which starts at 24 hours in the early stages of inflammation, and is only normalized when the inflammatory insult is resolved (Schliewert *et al*., 2022; Harvey, 2012).

Ângulo *et al*. (1997) described decreases in erythrocyte values, hemoglobin concentration, and hematocrit when they inoculated a mixture of equal parts of *Bothrops asper*, *Crotalus durissus durissus*, and *Lachesis muta stenophrys* venoms in horses producing antivenom serum. On the other hand, horses suffering from botropic accidents developed anemia attributed to coagulopathy and hemorrhagic diathesis, with a decrease in hematocrit, hemoglobin concentration, and erythrocyte count (Chiacchio *et al*., 2011). A second report, by Silva *et al*. (2011), showed a decrease in hematocrit on the 4th day after the bite, attributed to hemorrhagic edema and bleeding.

Hypoferremia was observed at the onset of the inflammatory response in horses, likely due to increased circulation of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) (Harvey, 2012; Frye, 2010). IL-6 and TNF-α were not measured in this study.

The horses in this study showed a significant increase in total leukocytes and segmented neutrophils, exceeding the maximum values of the reference range for these variables (Jain, 1986; Weiss and Wardrop, 2010) for the species, characterizing leukocytosis with neutrophilia after the three immunizations, regardless of the adjuvant used. Additionally, leukocytosis accompanied by mature neutrophilia indicates increased granulopoiesis in persistent inflammation in horses (Welles, 2010).

In a horse bitten by *Bothrops* spp., there was leukocytosis due to neutrophilia on the first day after the snakebite accident, which disappeared on the 4th day (Silva *et al*., 2011). Horses also poisoned by *Bothrops* spp. presented anemia and leukocytosis (Machado *et al*., 2019).

Metalloproteinases with disintegrin/cysteine-rich domains, like those found in jararagina, promote the release of inflammatory cytokines (IL-6, IL-1ß, and TNF-α), increasing circulating leukocytes and directly or indirectly affecting endothelial cells by increasing adhesion molecules, such as selectins, which results in an inflammatory condition (Clissa *et al*., 2006). Additionally, the proteolytic activity of metalloprotease BAP1 stimulates the migration of leukocytes to the site of venom application, mainly because BAP1 regulates the expression of selectins and induces the release of inflammatory mediators such as IL-1 and TNF (Fernandes *et al*., 2006). Therefore, proteins in the bothropic venom are essential for initiating the inflammatory response. Despite the significant decrease in the number of lymphocytes after the first and second immunizations, there was no lymphopenia when comparing the results with the reference range for horses (Jain, 1986; Weiss and Wardrop, 2010). Parra *et al*. (2009) did not

observe a significant difference in the number of lymphocytes in horses producing anticrotalid serum. Horses producing bothropic and crotalic antivenom did not show a significant difference in monocytes, eosinophils, and basophils, as observed in the horses in this study (Parra *et al*. 2009; Ângulo *et al*., 1997).

During the immunization protocol in this study, which began with Freund's incomplete adjuvant, followed by two immunizations with 4% aluminum hydroxide as adjuvants, the horses showed inflammatory reactions at the application sites. The transient changes in hematological variables and serum iron concentrations found in the animals in this study suggest that they were caused by the immune response resulting from the production of antivenom serum. These changes indicate the health of the animals based on the evaluation of hematological variables.

CONCLUSION

The immunization protocol employed in this study revealed that the application of the immunogen (venom plus adjuvant) caused local inflammatory reactions with the formation of abscesses, edema, and fistulas, and systemic inflammatory reactions observed through leukocytosis with neutrophilia, as well as hypoferremia, a sensitive indicator of inflammation in horses. However, by the end of the third immunization, the values of hematological variables and iron returned to normal.

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