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Liver function of mares submitted to *Bothrops* antigen inoculation for the production of anti-ophidian serum[1]

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ABSTRACT.- Del Castillo M.N., Ferreira C.S.C., Balaro M.F.A., Castanheira P., Almosny N.R.P & Pinna A.E. 2024. **Liver function of mares submitted to *Bothrops* antigen inoculation for the production of anti-ophidian serum.** *Pesquisa Veterinária Brasileira 44:e07276, 2024.* Departamento de Patologia Clínica, Universidade Federal Fluminense, Rua Vital Brazil Filho 64, Santa Rosa, Niterói, RJ 24230-340, Brazil. E-mail: mariana_nahum@hotmail.com

Ophidian accidents have been a medical and veterinary challenge. The only treatment available and recommended by the World Health Organization and the Ministry of Health is serotherapy. This study evaluated the liver function of equines submitted to the immunization protocol currently used by the "Instituto Vital Brazil" to produce anti-*Bothrops* serum. Five equines were evaluated with *Bothrops* venom inoculation during three immunization cycles. Blood samples were taken for a biochemical test and a liver assessment due to the damage caused to the liver. The biochemical data that presented significant differences in all animals compared to baseline were AST, ALT, GGT, FA, GLDH and serum protein. Creatinine did not change throughout the experiment. The results obtained suggest that liver alterations may occur throughout the three immunization cycles. There is a great need for new studies aimed at broadening knowledge about venom inoculation protocols in serum producer equines and minimizing the adverse effects of immunization.

INDEX TERMS: Equine, anti-ophidian serum, liver, GLDH.

RESUMO.- [Função hepática de éguas submetidas à inoculação de antígeno botrópico para produção de soro antiofídico.] Acidentes ofídicos têm sido um desafio médico e veterinário. O único tratamento disponível e preconizado pela Organização Mundial da Saúde e pelo Ministério Saúde é a soroterapia. O presente estudo avaliou a função hepática dos equinos submetidos ao protocolo de imunização empregado atualmente pelo Instituto Vital Brazil para produção do soro antibotrópico. Foram avaliados cinco equinos com inoculação de veneno Botrópico durante o período de três ciclos de imunizações. Amostras de sangue foram coletadas para realização de exame bioquímico e avaliação hepática por conta das lesões causadas no fígado. Os dados bioquímicos que apresentaram diferenças significativas, em todos os animais, comparados ao tempo basal, foram AST, ALT, GGT, FA, GLDH e proteína sérica. Creatinina não apresentou alteração ao longo do experimento. Os resultados obtidos sugerem que ao longo dos três ciclos de imunização podem ocorrer alterações hepáticas. Há uma grande necessidade de novos estudos com objetivo de ampliar o conhecimento acerca dos protocolos de inoculação de veneno nos equinos soroprodutores e minimizar os efeitos adversos da imunização.

TERMOS DE INDEXAÇÃO: Equinos, soro antiofídico, fígado, GLDH.

INTRODUCTION

Anti-ophidian serotherapy has been used for over 120 years to treat accidents caused by snakes and other venomous animals. In 1894, three researchers began anti-ophidian serotherapy: Albert Calmette (Calmette 1894), Césaire Phisalix, and Gabriel Bertrand (Phisalix & Bertrand 1894). These researchers reported on the use of anti-ophidian serum based on the venom of the *Naja tripudians*, the France Viper, and *Pseudechis* from Australia.

Technological advances in the field of antivenom production were focused on the processing of hyperimmune plasma and the purification and fractionation of immunoglobulins, while immunization protocols made little progress during

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this period (Alvarenga et al. 2014). Serums obtained from hyperimmune horse plasma are a mainstay in the treatment of accidents caused by venomous snakes. The production of serum involves the subcutaneous injection of crotalid venoms into horses. Although these injections cause local alterations such as edema and abscesses in a small percentage of animals, systemic alterations have not yet been demonstrated. The venom inoculated into horses is metabolized by the liver and eliminated by the kidneys (Iwanaga & Suzuki 1979, Mebs & Ownby 1990).

There are few studies evaluating hepatic and renal alterations in animals used in the production of antivenoms. The diagnosis of liver disease is made based on clinical signs, laboratory analyses and imaging tests, as well as histopathological examination of a fragment of the organ taken by biopsy (Davoudi et al. 2013). Increases in serum concentrations of the enzymes aspartate aminotransferase (AST), sorbitol dehydrogenase (SDH) and glutamate dehydrogenase (GLDH) indicate hepatocyte damage in equines (Lester et al. 2015). This study aimed to evaluate the liver function of equines submitted to the immunization protocol currently used by the "Instituto Vital Brazil" (IVB) to produce anti-*Bothrops* serum.

MATERIALS AND METHODS

Animal Ethics. The project was approved by the CEUA of the "Instituto Vital Brazil" (IVB) under number 007/2015.

Animals. The experiment used five female equines without a defined breed, ranging in age from 5 to 15 years old, which had not previously been inoculated with poison and housed at the Instituto Vital Brazil (IVB) under semi-intensive management. All the animals were fed in individual troughs and received 4kg of feed a day, 12kg of chopped grass, 50g of mineral supplement and water at will. The mares (n=5) were subjected to three immunization cycles with a mixture of *Bothrops* venom.

***Bothrops* venom.** The venom used in the immunizations was prepared by mixing five species of the *Bothrops* genus: *B. jararaca* (50%), *B. jararacussu* (12.5%), *B. neuwied*i (12.5%), *B. moojeni* (12.5%), and *B. alternatus* (12.5%). The mixture was the same as that used in the immunization protocols to obtain the hyperimmune plasma used to produce anti-ophidian serum at the IVB.

Immunization of equines. The first immunization cycle was called baseline immunization and lasted eight weeks, with one inoculation per week. The first inoculation consisted of 5mg of *Bothrops* venom diluted in 5ml of saline solution plus 5ml of complete Freund's adjuvant. The second inoculation consisted of 5mg of venom diluted in 5ml of saline solution (8.5%) plus 5ml of montanide adjuvant ISA50V2. The other inoculations (4th to 8th) were of 5mg of *Bothrops* venom diluted in 10ml of saline.

After a 4-week rest without immunization, the animals were submitted to a second immunization cycle lasting two weeks. In the first week, an inoculation consisting of 6mg of venom diluted in 4ml of saline plus 4ml of montanide adjuvant ISA50V2 was carried out. In the second week, three inoculations were made, 48 hours apart, consisting of 2mg of venom diluted in 8ml of saline. The animals underwent another rest period of four weeks without immunization and were then subjected to the third immunization cycle, which followed the same protocol as the second immunization cycle (Fig.1).

Complementary tests. During the immunization period, blood samples (with and without anticoagulant) were taken once a week and ultrasound scans were carried out every two weeks to assess the liver. For the control, a blood sample was taken, and an ultrasound assessment was made of each equine one week before (week 0) the start of the first immunization cycle. This sample was identified as S0. Thirteen blood samples and seven ultrasound evaluations were obtained from each equine and identified according to the week in which they were collected (S0 to S20).

Blood sample. After local antisepsis, the blood samples were obtained by puncturing the jugular vein, using a vacutainer system (25 X 8mm needles) and siliconized glass tubes for vacuum collection, with a capacity of 5.0ml containing 15% ethylenediamidetetraacetic acid (EDTA) as an anticoagulant. Immediately after collection, the samples were carefully homogenized and stored in a refrigerated container suitable for transport. Then, to perform the biochemical and total protein tests, blood was collected in a sterile 10ml vacuum tube without anticoagulant. The refrigerated blood samples were transported to the Marcilio D. Nascimento Clinical and Molecular Research Laboratory of the Veterinary Faculty of the "Universidade Federal Fluminense" (UFF). The EDTA-containing blood samples taken from the equines at the "Instituto Vital Brazil" Farm, which were submitted to the anti-*Bothrops* hyperimmunization protocol, were used to carry out laboratory tests included a complete blood count (CBC) consisting of an erythrogram (count of the number of erythrocytes), hemoglobin, globular volume and red blood cell indices: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular concentration (MCHC), and leukogram (global count). Blood samples without anticoagulants were used for liver biochemistry tests, measuring the enzymes Aspartate Transaminase Aminotransferase (AST), Alanine Transaminase (ALT), Gamma Glutamyl Transferase (GGT), Alkaline Phosphatase (AF), Glutamate Dehydrogenase (GLDH) and Total Protein. A spectrophotometric or kinetic methodology was adopted, according to the manufacturer's standards, using automated equipment (Labmax 4000®, São Paulo, Brazil) and commercial kits (LabTest®, São Paulo, Brazil).

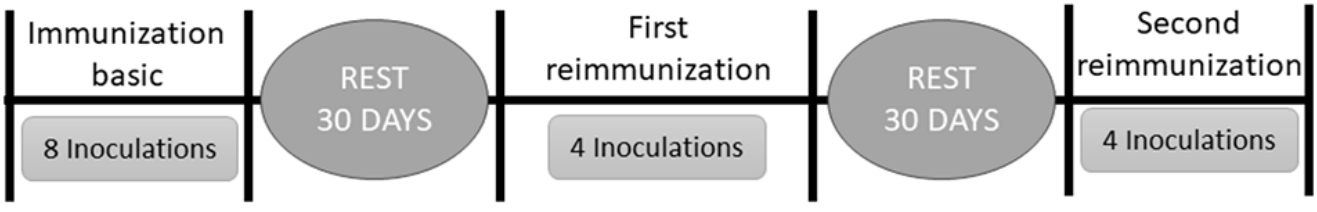

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Fig.1. Three-cycle immunization protocol for *Bothrops* venom used by the "Instituto Vital Brazil" in equines.

Ultrasound evaluation. For the ultrasound examination, an ultrasound machine (Sonoscape, model S6V) with a convex transducer with a frequency of 2 to 5MHz was used. Ultrasound evaluations were carried out by dividing the animal's abdomen into delimited areas called ultrasound windows, which are separated as follows: middle third of the costal arch on the left side between 8° and 13° ICS; middle third of the left costal arch between 12° and 15° ICS; ventral region of the caudal abdomen to the xiphoid cartilage and external; middle third of the costal arch on the right side, between the 10° and 12° ICS. The ultrasound images of the liver were evaluated for morphology, architecture, and contour.

Statistical analysis. The results were analyzed using the Statistical Analysis System (BIOESTAT® 5.0, Instituto Mamirauá, Amazonas, Brazil). A descriptive analysis was carried out, and the data was tabulated as mean ± standard deviation or frequency (%). All quantitative data was assessed for normality using the Lilliefors test. The analysis of variance (ANOVA) was applied for paired measurements, and means were compared using the Tukey test (when CV ≤30%) or the Student's LSD T-Test (when CV >30%). Non-normal data was evaluated using the Kruskal-Wallis test, and means were compared using the Dunn test. Categorical data was evaluated using Fisher's exact test. For all the data, a significant difference was considered when $P<0.05$.

RESULTS

The physical examination presented no significant alterations, apart from the occurrence of abscesses in the muscles close to the site of venom inoculation, which were more frequent in the weeks of the first immunization cycle compared to the other weeks.

The blood count presented a decrease in erythrocytes in S1 and S4 and an increase in S8. Hemoglobin decreased in S4, rising again in S5, and remaining there until the end of the experiment. The hematocrit, MCV, and MCHC parameters demonstrated no significant changes, unlike the MHC, which showed a decrease in serum values in S13, S14, and S20. There was an increase in leukocyte values in S7 and a peak with values higher than the reference values in S13, remaining high until the end of the experiment.

In biochemical tests, apart from serum albumin and creatinine (Fig.2), there was a time effect (baseline to S20) on serum AST, ALT, GGT, FA and GLDH ($P<0.05$). Serum AST rose from S5 and remained stable until the end of the study. ALT presented a peak at S3 of the study and then a decrease in its enzymatic activity between S8 and S14 of the study (Fig.3). GGT presented a first peak of serum elevation between S5 and S8 and a second peak between S13 and S20. AF presented higher serum activity at S5 and S20 (Fig.4). The GLDH enzyme presented higher serum activity between S5 and S8 (Fig.5). There was then a decrease in values in the final two weeks (S19 and S20) of the study. There was also an increase in total serum protein values from S5 onwards, which remained stable until the end of the study at S20 (Table 1).

The ultrasound images of the kidneys demonstrated no statistical difference. On the other hand, ultrasound images of the right hepatic lobe presented significant changes in contour in S2 and S8, returning to normal at the end of the experiment and in echotexture, which was heterogeneous in S20 ($P<0.05$). The left hepatic lobe, on the other hand, presented changes in contour and echogenicity at S4 and S14, respectively ($P<0.05$).

DISCUSSION

Edema developed near the inoculated area in the process of immunizing the mares, results also found by Sousa et al. (2011), who administered the venoms of *Bothrops jararaca*, *Bothrops jararacussu, Bothrops moojeni,* and *Bothropoides neuwiedi* subcutaneously in six equines. This tumefaction has also been described in both natural cases (Méndez & Riet-Correa 2007, WHO 2007) and experimental cases (Soerensen et al. 1995, Caldas et al. 2008, Aragão et al. 2010, Magalhães 2019) by snakes of the former *Bothrops* genus, regardless of the

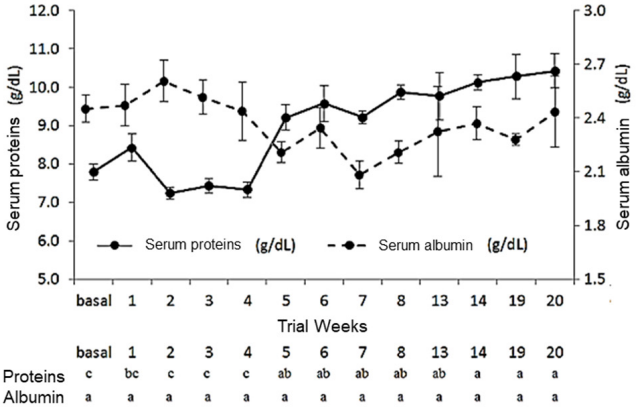

Fig.2. Evaluation of total protein and serum albumin in equines throughout the experimental period. Total proteins demonstrated an effect of time, with an increase starting at S5 and remaining stable until the end of the study at S20. Albumin, on the other hand, demonstrated that there was no effect of time between baseline (S0) and the last week of collection (S20).

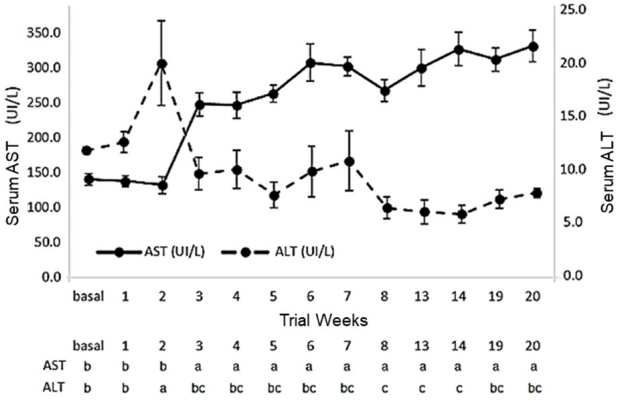

Fig.3. Evaluation of the serum enzymes Aspartate Transaminase Aminotransferase (AST) and Alanine Transaminase (ALT) in equines throughout the experimental period, demonstrating the effect of time between baseline (S0) and the last week of collection (S20). Serum AST rose from S5 and remained stable until the end of the study. ALT, on the other hand, presented a peak at S3 of the study and then a decrease in its enzymatic activity between S8 and S14.

species affected. One probable cause is the intense proteolytic action (metalloproteases) of the venom with a subsequent generalized inflammatory process, causing edema and even local necrosis. According to Castrillón-Estrada et al. (2007), the onset of symptoms occurs between 10-20 minutes after envenomation with local edema, and the venom of newborn snakes has a greater hemorrhagic, edematous, and lethal effect compared to adult snakes.

At the end of the first immunization cycle (S8), there was an increase in the serum activities of the enzymes AST and GLDH, which, according to Lester et al. (2015), indicate hepatocyte damage in equines. In the study by Armengou et al. (2013), data was collected on sick foals and the relationship with some biochemical evaluations, and it was observed that foals that did not survive had a higher median plasma GLDH concentration than the survivors, which may be related to acute liver damage. The necropsy revealed mild microscopic liver lesions, such as hemorrhage, inflammatory infiltration, fibrosis, or hepatocellular necrosis, corroborating the elevated GLDH in the foals. The increase in serum AST enzyme activity persisted in both the second and third cycles, as reported by Barbosa et al. (2011), who assessed sensitivity to *Crotalus durissus terrificus* venom among buffalo and cattle. AST is a leaky enzyme that is found in high concentrations in hepatocytes and skeletal and cardiac muscle cells of all species. It is, therefore, not a liver-specific enzyme, but its elevation can reveal damage to hepatocytes or muscles (Eades 2009, Thrall et al. 2014).

The increase in serum ALT activity may be related to the muscle damage caused by *Bothrops* venom, as described in the literature (Castrillón-Estrada et al. 2007). Albumin presented a reduction from baseline to the end of the first cycle, which was maintained until the end of the third cycle, corroborating with an experiment by Takahira (1996), in which *Bothrops jararaca* venom was inoculated into dogs. The total protein value was high due to the increase in globulin synthesis resulting from the immunization process since globulins represent a group of large proteins and encompass various types of antibody molecules (Mcgowan 2008). FA and GGT are induction enzymes considered to be indicators of cholestasis

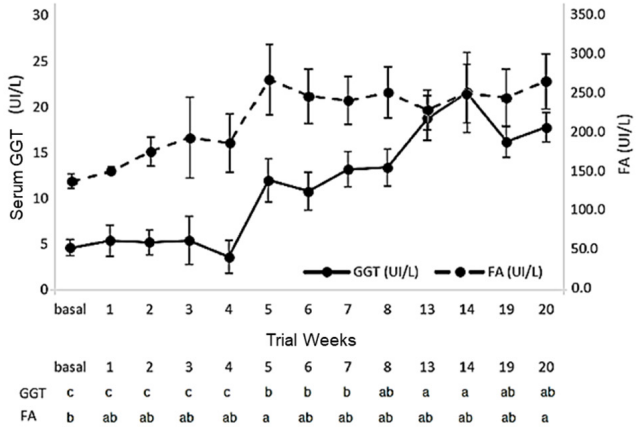

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Fig.4. Evaluation of serum enzymes Gamma Glutamyl Transferase (GGT) and Alkaline Phosphatase (AF) in equines throughout the experimental period, demonstrating the effect of time between baseline (S0) and the last week of collection (S20). GGT presented a first peak of serum elevation between S5 and S8 and a second peak between S13 and S20. AF presented higher serum activity at S5 and S20.

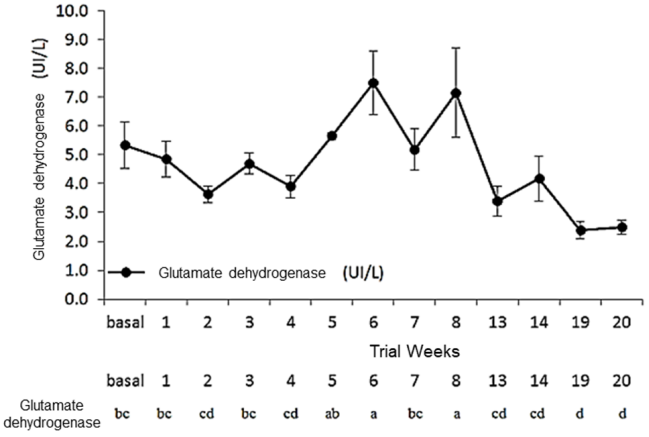

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Fig.5. Values found for the enzyme Glutamate Dehydrogenase (GLDH) in equines throughout the experimental period, demonstrating the effect of time between baseline (S0) and the last week of collection (S20). The GLDH enzyme presented higher serum activity between S5 and S8. Then, there was a decrease in values in the final two weeks (S19 and S20).

Table 1. Mean ± standard deviation of the index evaluated by serum biochemistry at baseline (before the start of the immunization cycle), after the 1st, 2nd and 3rd immunization cycles of the experimental animals

Serum biochemical index	Baseline	After 1st cycle of immunizations	After 2nd cycle of immunizations	After 3rd cycle of immunizations	Total
Urea (mg/dL)	34.0a ± 3.4	26.6b ± 2.7	30.0ab ± 4.2	30.0ab ± 1.2	28.8 ± 4.2
Creatinine (mg/dL)	1.3 ± 0.2	1.3 ± 0.3	1.3 ± 0.4	1.3 ± 0.3	1.3 ± 0.3
Total protein (g/dL)	7.8b ± 0.5	9.9a ± 0.4	10.1a ± 0.5	10.4a ± 1.0	9.0 ± 1.3
Albumin (g/dL)	2.5 ± 0.2	2.2 ± 0.1	2.4 ± 0.2	2.4 ± 0.4	2.4 ± 0.3
AST (IU/L)	140.4b ± 18.4	267.4a ± 34.5	326.4a ± 51.6	330.6a ± 49.9	254.7 ± 78.9
ALT (IU/L)	12.0a ± 0.7	6.6b ± 2.3	6.0b ± 1.9	8.0b ± 1.0	9.8 ± 5.1
GLDH (UI/L)	5.3b ± 1.8	7.2a ± 3.4	4.2ab ± 1.7	2.5ab ± 0.5	4.6 ± 2.1
GGT (IU/L)	4.6c ± 1.9	13.4b ± 4.4	21.6a ± 9.6	17.8ab ± 3.6	11.4 ± 7.4
FA (IU/L)	139.0b ± 20.8	252.0a ± 71.8	250.6a ± 82.5	266.6a ± 77.5	219.5 ± 78.3

[a,b] Distinct letters between columns indicate a significant difference according to the Tukey test (P<0.05).

in equines (Lester et al. 2015) and presented an increase in serum activity over the three cycles. The increase in this activity was probably due to intrahepatic causes because of the inoculation of the antigen in the immunization process.

According to Belluomini et al. (1982), cattle react most sensitively to snake venom, followed by equines, ovines, goats, canines, and swine. According to Rosenfeld (1971), carnivores seem to be more resistant to ophidian venoms than other animals, but the cat is resistant to *Bothrops* venom (proteolytic and coagulant) and very sensitive to the venom of South American *Crotalus* spp.; the hamster is more resistant than the cat to crotalic venom.

The results of the hematological tests showed normochromic normocytic anemia and persistent thrombocytopenia, corroborating the findings of Sousa et al. (2011), probably caused by the exploratory bleeding process during the second and third immunization cycles. Oliveira et al. (2004) found changes in the blood profile characterized by leukocytosis in five cows inoculated with *Bothrops* venom, similar to the findings of this experiment. Otero et al. (1992) and Cardoso et al. (2009) cited leukocytosis as a typical laboratory finding in human crotalic envenomations. Magalhães (2019) also reported the presence of thrombocytopenia and leukocytosis with left-shifted neutrophilia and mild anemia in humans in the first few hours after the bite. In a case report, Tolentino et al. (2019) observed the presence of liver coagulation disorders (thrombocytopenia, hemorrhage, and increased blood coagulation time; decrease in albumin and globulin) and muscle damage (considerable increase in lactate dehydrogenase and creatine kinase), partially corroborating the findings of our experiment.

It was noted that the ultrasound examination was important for the early detection of alterations in the liver parenchyma, even in the absence of changes in biochemical tests, in agreement with the reports described by Mamprim et al. (1997) in the experiment with dogs. The lack of literature on hepatic ultrasound examinations, especially in equines, makes it difficult to compare with the present study and determines the importance of our ultrasound findings demonstrating suggestive alterations of an acute condition. It is, therefore, understood that ultrasound is essential for identifying abnormalities in the liver parenchyma at an early stage.

CONCLUSION

Significant serum biochemical alterations are detected in equines inoculated with *Bothrops* venom even in the absence of symptoms, which may suggest acute lesions throughout the three immunization cycles. There is an increase in the enzymatic activity of AST, GLDH, GGT and FA throughout the immunization process, which suggests hepatotoxicity action in equines. Total serum proteins also rise as a result of the production of immunoglobulins. The ultrasound images show changes in the integrity of the liver and kidney over the three immunization cycles evaluated. When correlating the results of the laboratory tests with the ultrasound images, it can be seen that both present alterations from the first immunization cycle, such as anemia and irregular liver contour. Thus, due to animal welfare issues and tissue damage resulting from immunization in equines, more studies are needed on *Bothrops* venom inoculation protocols in order to minimize the adverse effects of immunization.

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Conflict of interest statement.- The authors declare that there is no conflict of interest.

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