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CLINICAL AND LABORATORIAL EFFECTS AND IMMUNOGENICITY OF *Loxosceles* spp. VENOM PROTEINS IN HORSES PARTAKING IN ANTIVENOM PRODUCTION

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Tese apresentada à Escola de Veterinária da Universidade Federal de Minas Gerais como requisito para a obtenção do grau de Doutora em Ciência Animal.

Área: Medicina e Cirurgia Veterinárias.

Orientador: Prof. Dr. Benito Soto Blanco
Co-orientador: Prof. Dr. Carlos Delfin Chávez Olórtegui
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RESUMO

O envenenamento por aranhas detém importância mundial no âmbito da saúde pública e alcança números cada vez mais significativos a cada ano. Acidentes envolvendo *Loxosceles* spp. detêm destaque nessa estatística, especialmente no Brasil. A soroterapia permanece sendo o único tratamento efetivo contra esse agravo e o seu processo produtivo enfrenta diversos problemas, uma vez que o mesmo é diretamente dependente da imunização de animais (principalmente cavalos) para o posterior processamento do plasma, o que possui implicâncias éticas referentes à bem estar animal. Dessa forma, o objetivo do presente trabalho é avaliar os efeitos clínicos causados pelo veneno de *Loxosceles* spp. em equinos submetidos a imunização para produção de antiveneno, assim como o de coelhos submetidos a um procedimento de imunização semelhante. No experimento 1, 11 equinos hígidos, nunca antes imunizados, foram avaliados em três momentos experimentais distintos: T0 (antes da imunização); T1 (após o primeiro ciclo de imunização); e T2 (após a primeira sangria comercial). Os animais foram avaliados clinicamente, submeteram-se à coleta de sangue e ao exame eletrocardiográfico (ECG). Achados clínicos de destaque recaíram sobre a presença de abscessos subcutâneos supurados atribuídos ao emprego de adjuvantes de Freund e tromboflebites devido às venopunções sistemáticas. Equinos em T2 apresentaram as alterações hematimétricas mais relevantes, com redução do hematócrito (PCV), contagem de células vermelhas (RBC) e de hemoglobina. Funções hepática e renal permaneceram normais. A hiperproteinemia diagnosticada foi atribuída ao aumento do teor de globulinas, O ECG ilustrou arritmias em poucos equinos em T2, como bloqueio átrio-ventricular de segundo grau (BAV-2) e aumento em amplitude de ondas T e R. No experimento 2, sete equinos, os quais já haviam sido submetidos à seis ou mais ciclos completos de imunização para produção de antiveneno, foram avaliados e denominados grupo imunizado (GI). Onze equinos, sob o mesmo manejo, foram dispostos como grupo controle (GC). GI apresentou menor PCV e RBC, apesar de mantê-los dentro dos índices de referência para a espécie. A função renal não se apresentou comprometida, mas as enzimas hepáticas apresentaram-se elevadas em relação ao GC, provavelmente devido ao alto requerimento de produção de imunoglobulinas. O ECG ilustrou anormalidades de traçado no GI, corroboradas com o aumento na enzima creatina quinase em sua fração MB (CK-MB). No experimento 3, 11 coelhos machos Nova Zelândia foram utilizados. Cinco foram alocados no grupo controle (GC), recebendo adjuvante (montanide) e tampão salina-fosfato. Os seis coelhos restantes receberam 21µg de veneno de *Loxosceles* spp. empregando como adjuvante o montanide (GV). Após cinco ciclos de imunização, um desafio foi realizado com 7µg de veneno de *L. intermedia*, com posterior mensuração das lesões dermonecróticas. Os coelhos foram eutanasiados e amostras de órgãos e tecidos foram coletados para análise histopatológica. Nenhum parâmetro sanguíneo referente ao eritrograma mostrou-se alterado significativamente, mas a contagem total de leucócitos foi maior em GV, corroborando com a ação quimiotáxica neutrofílica já descrita para o veneno. Fígado e rins mantiveram a função preservada de acordo com a análise de bioquímica sérica. O ECG não demonstrou alterações entre grupos e momentos experimentais. A

histopatologia elucidou uma tendência do veneno loxoscélico em induzir cardio/reno e hepatotoxicidade, tanto de forma direta como indireta. Conclui-se que apesar de o veneno possui ação cardio/reno e hepatotóxica, ambos coelhos e equinos mantiveram-s em condições clínicas adequadas. Uma maior deve ser dada aos equinos no período pós-sangria, devendo os mesmos serem acompanhados. A reinfusão de papa de hemácias é uma sugestão para esses animais.

Palavras-chave: aranha-marrom; cardiovascular; clínica; equino, soro; tóxico

ABSTRACT

Spider envenomation holds worldwide importance in public health and reaches significant and increasing numbers every year. *Loxosceles* spp. plays an important role in these statistics, especially in Brazil. Antivenom remains the only effective treatment against this ailment, and its production faces several hindrances since it depends on immunizing animals (mainly horses) and later processing their plasma, which leads to increasing animal welfare concerns. Therefore, the aim of this study is to evaluate the general clinical effects of *Loxosceles* spp. venom in horses that underwent immunization protocols for loxoscelic antivenom procurement, as well as those in rabbits who underwent a shorter immunization protocol. In experiment 1, eleven healthy horses, never immunized, were evaluated on three different periods: T0 before immunization; T1 after their first loxoscelic antivenom immunization; and T2 after their first commercial bleeding. Horses were clinically evaluated, sampled for blood, and underwent electrocardiographic (ECG) recordings. Significant clinical findings were the several suppurated subcutaneous abscesses due to the use of Freund's adjuvants and thrombophlebitis due to systematic venipunctures for commercial bleeding procedures. Horses at T2 presented the most blood alterations, including reduced packed cell volume (PCV), red blood cells (RBC), and hemoglobin. Liver and renal functions were unaffected. Hyperproteinemia occurred due to increase in globulin levels. ECG showed arrhythmias in few horses in T2, such as second-degree atrioventricular block (AV-block), as well as an increase in T and R waves. On experiment 2, seven crossbred horses, who had partaken in six or more complete antivenom-producing cycles, were used and established as the immunized group (IG). Eleven horses, under the same handling and general management, were established as the control group (CG). IG presented lower red blood cell count and packed cell volume, despite keeping values within inferior limits for the species. Renal function was not impaired, but liver-related enzymes were higher when compared to CG, probably due to liver exertion from immunoglobulin synthesis. ECG showed some abnormalities in IG, corroborated by increase in creatine kinase/isoenzyme MB fraction (CK-MB). In experiment 3, eleven male New Zealand rabbits were used. Five were allocated as a control group (CG), that received adjuvant (montanide) and phosphate-buffer saline. The six remaining rabbits received 21μg of *Loxosceles* spp. venom using montanide as adjuvant (VG). After five immunization cycles, a trial with 7μg of *L. intermedia* was performed, and dermonecrotic lesions were measured. Rabbits were then euthanized, and their organs harvested for histopathology analysis. No erythrocyte-related parameter showed significance, but white blood cell count was higher in VG, corroborating with venom's neutrophil chemotaxis. Liver and kidney functions were also preserved according to blood biochemical panel. ECG showed no alteration between experimental groups and evaluation periods. Histopathology showed a tendency for loxoscelic venom to produce a direct and indirect cardiotoxicity, renal toxicity, and hepatotoxicity. It was concluded that despite loxoscelic venom exerts cardiotoxicity, hepatotoxicity, and renal toxicity, both rabbits and horses were kept within acceptable clinical conditions. A crucial point to be highlighted is bleeding for industrial antivenom production, when horses should receive greater attention and perhaps reinfusion with suspended red blood cells.

Keywords: brown spider, cardiovascular, clinical, equine, serum, toxic.

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LIST OF ABBREVIATIONS

%	Per cent
°C	Degree Celsius
µg	microgram
µL	microliter
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AMPs	Antimicrobial peptides
APTT	Activated partial thromboplastin time
AST	aspartate transaminase
AV	Atrio-ventricular
bpm	Beats per minute
C1P	Ceramide-1-phosphate
CCPI	Center of Production and Research of Immunobiologicals
CEUA	Ethical Committee for the Use of
CG	Control group
CK	creatine kinase
CK-MB	creatine kinase/isoenzyme MB fraction
CRT	Capillary refill time
CXCL-1	C-X-C Motif Chemokine Ligand 1
CXCL-2	C-X-C Motif Chemokine Ligand 2
ECG	Electrocardiography
EDTA	Ethylenediaminetetraacetic acid
EST	expressed sequence tag
fL	fentoliter
GGT	gamma-glutamyl transpeptidase
GM-CSF	granulocyte macrophage colony-stimulating factor
HE	Hematoxilin-Eosin
ICK	Inhibitor cystine knot
IG	Immunized gropu
IL-6	interleukin-6
IL-8	interleukin-8
kDa	Kilodalton
kDa	kilodalton
Kg	Kilogram
L	liter
LDH	lactate dehydrogenase
LPA	lysophosphatidic acid

MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCP-1	monocyte chemoattractant protein-1
MCV	Mena corpuscular volume
mg	miligram
mL	mililiter
mm	milimiter
MMP2	Type 2 metalloprotease
MMP7	Type 7 metalloprotease
MMP9	Type 9 metalloprotease
MND	Minimum necrotizing dose
mpm	Movements per minute
MPV	mean platelet volume
ms	milisecond
mV	milivolt
nm	nanometer
OTH	neutrophils, monocytes and basophils
P.A.S.	Periodic Acid Schiff
PBS	Phosphate-buffer saline
PCV	packed cell volume
PDW	platelet distribution width
PGE2	Prostaglandin E2
P-LCR	platelet clump
PLD	Phospholipase-D
PLT	total platelet count
ppm	Pulse per minute
PR	Paraná State
RBC	red blood cell count
RDW	red blood cell distribution width
sec	second
SEM	Standard error
TCTP	Translationally controlled tumor protein
TP	total proteins
UFMG	Federal University of Minas Gerais
V	Volts
VG	Venom group
WBC	white blood cell count

SUMMARY

INTRODUCTION

Spider envenomation holds worldwide importance in public health and reaches significant and increasing numbers every year. Despite not possessing an aggressive behavior, spiders from the *Loxosceles* genus, popularly known as “brown spiders”, play a prominent role in those statistics, especially in Latin America (da Silva et al., 2004; Rodríguez and Méndez, 2008). Their bite produces a clinical syndrome called loxoscelism, characterized by several clinical manifestations, such as dermonecrotic lesions with gravitational spreading, renal failure and hematological disturbances (Futrell, 1992; Swanson and Vetter, 2006). In Brazil, spider bites from *Loxosceles* spp. are recognized as a public health issue since 1957 (Rosenfeld et al., 1957), and there are at least 17 species distributed through all its geographic regions (Platnick, 2020; Bertani et al., 2018). It is the most relevant form of araneism in the country (Ministério da Saúde, 2019), due mainly to its high rate of household infestation (Ribeiro et al., 1993).

The venom of the brown spider is defined by Senff-Ribeiro et al. (2008) as a complex mixture of toxins enriched by low molecular mass peptides. However, its mechanism of action has not yet been totally elucidated (Silva et al., 2004; Chaim et al., 2011). Crude venom has many components, which include phospholipases, hyaluronidases, serine proteases, metalloproteases, venom allergens and ribonucleotide phosphohydrolase (Futrell, 1992; Oliveira et al., 2005; Gremski et al., 2014; Oliveira-Lima et al., 2016; Langenegger et al., 2019). Phospholipase D is the most studied toxin in *Loxosceles* spp. venom, since it is the main responsible for the venom’s biological effect, which encompasses impacts in cellular growth, proliferation and differentiation of several host cell types (Paludo et al., 2006; Corrêa et al., 2016). Therefore, this venom will promote neutrophilic infiltration, complement system activation, platelet aggregation, edema, increased vascular permeability, hemolysis, and renal failure (Forrester et al., 1978; Rodríguez and Mendéz, 2008; Chaves-Moreira et al., 2011).

Specific antivenom remains the primary medical resource for treating *Loxosceles* envenomation (Hogan et al., 2004; Pauli et al., 2006), and horses are known as the main species for commercial procurement of antiserum against several toxins. This is mainly due to their easy handling and adaptability to different climates and their ability to provide

greater serum volumes for antiserum production. Furthermore, their antibodies purification protocols hold higher standards both in laboratory and commercial fields (Pratanaphom et al., 1997; Waghmare et al., 2014), thus optimizing the laborious process of procuring high quality and efficient antivenom (Baptista et al., 2012). Figueiredo et al. (2014) highlight that hyperimmunization protocols in order to produce *Loxosceles* antivenom can cause several impacts on horses, such as ulcers, abscess, and renal failure. However, no further studies have focused on the true clinical impact on horses' health, not only regarding the systemic effects of the venom, but also on the impact of partaking in several and consecutive immunization protocols, which consist of animals undergoing systematic bleedings, physical restraint, and stress.

Therefore, the aim of this study is to evaluate the clinical effects on horses that underwent immunization protocols for the procurement of *Loxosceles* spp. antivenom.

OBJECTIVES

General

Evaluate the general clinical effects of *Loxosceles* spp. venom in horses that underwent immunization protocols for loxoscelic antivenom procurement.

Specific

- Evaluate the clinical impacts over the general physical examination of naïve horses partaking in their first loxoscelic antivenom production protocol.
- Evaluate hematological and biochemical alterations of horses before and during the participation of their first loxoscelic antivenom production protocol.
- Diagnose the development of arrhythmias in naïve horses during their loxoscelic immunization process.
- Identify the clinical impacts of the immunization protocol in horses that have partaken in several loxoscelic antivenom production protocols throughout their lives.
- Diagnose permanent or transient hematological and biochemical alterations in horses that have undergone several loxoscelic immunization protocols.
- Perform electrocardiography in horses that have undergone multiple loxoscelic immunization protocols, thus diagnosing and establishing the severity of arrhythmias.
- Compare clinical, hematological, biochemical and electrocardiographic results of horses partaking on their first loxoscelic immunization protocol and those who had partaken in many.
- Evaluate hematological and biochemical impacts of the immunization protocol using *Loxosceles* spp. venom in rabbits.
- Evaluate electrocardiography alterations in rabbits that have undergone loxoscelic immunization protocols.
- Evaluate pathological alterations in organs such as heart, lungs, spleen, liver, kidneys, and skin on rabbits that underwent loxoscelic immunization protocol.

CHAPTER 1

Review – *Loxosceles* spp. venom and its mechanism of action

Loxosceles genus

Loxosceles spiders are the only globally distributed arachnid species with the ability to cause necrotizing skin lesions. Spiders from the *Loxosceles* genus belong to the Sicariidae family, Labidognatha sub-order, Araneida order, Arachnida class, and Arthropoda phylum. Popularly known as “brow spiders” or “brown recluse spiders”, they possess a brownish color ranging from fawn to dark, and a violin-shaped marking on the dorsal aspect of their cephalothorax. Their size can vary from 1 to 5 cm in diameter. The most efficient identification method, however, is based on the U shape format of its six eyes, arranged in three diads. These spiders are sedentary, non-aggressive, and nocturnal. Accidents usually occur in the warmest months of the year, encompassing spring and summer (Silva et al., 2004; Rodríguez e Méndez, 2008; Chaim et al., 2011; Bertani et al., 2018).

Loxosceles envenomation can cause a syndrome named loxoscelism, with two clearly defined clinical forms: cutaneous or viscero-cutaneous. Cutaneous loxoscelism occurs in the majority of cases, whereas viscero-cutaneous manifestations occur to a lesser extent, but a higher fatality rate. Cutaneous loxoscelism is characterized by dermonecrotic lesions with a typical presentation of a livedoid plate and gravitational spreading. These lesions very often evolve to necrotizing wounds. Patients with viscero-cutaneous loxoscelism can manifest fever, weakness, vomiting, intravascular hemolysis, vascular alterations, and renal failure (Hogan et al., 2004; Silva et al., 2004; Swanson and Vetter, 2006; Chaim et al., 2011; Bertani et al., 2018).

Prevalence of loxoscelism as a health problem in several Latin American countries, including Brazil, have promoted increased interest in studies of brown spider venom in order to expand knowledge regarding the pathophysiology of loxoscelism. In Brazil, spider bites from *Loxosceles* spp. are recognized as a public health issue since 1957 (Rosenfeld et al., 1957). The country houses at least 17 species distributed through all its geographic regions (Bertani et al., 2018; Platnick, 2020), with most accidents attributed

to *Loxosceles gaucho*; *L. intermedia* and *L. laeta*. It is the most relevant form of araneism in the country (Ministério da Saúde, 2019), due mainly to its high rate of household infestation (Ribeiro et al., 1993). Other species, such as *L. rufescens* and *L.reclusa*, also play an essential role in human envenomation, especially in North American countries (Hogan et al., 2004; Swanson and Vetter, 2006; Gremski et al., 2014; Sampaio et al., 2016; Oliveira-Mendes, 2019).

Loxosceles venom

Loxosceles venom is a complex mixture of proteins and peptides with a peptide molecular mass ranging from 2 to 40 kDa, encompassing numerous protein molecules with toxic or enzymatic activity acting synergistically (Chaim et al., 2011; Gremski et al., 2014) Venom may have variated effects, depending on the species, or even between males and females of the same species. Venom from *Loxosceles laeta*, for example, retains greater hemolytic and dermonecrotic effects than those of *Loxosceles intermedia*, whereas female venom appears to be more toxic than that of male specimens (Oliveira et al., 2005; Oliveira-Lima et al., 2016).

Transcriptomic analyses have been providing a more profound understanding on loxoscelic venom composition (Langenegger et al., 2019) and highlighting its potential as biotools in the development of novel therapeutics (Chaim et al., 2011; Chaves-Moreira et al., 2019). Fernandes-Pedrosa et al. (2008) examined transcripts in venom glands of *L. laeta* and found that 16.4% of total expressed sequence tags (ESTs) belonged to recognized toxin-coding sequences, including Phospholipase-D and neurotoxins, with an obvious predominance of Phospholipase-D as the most abundant transcript. Possible toxins, such as metalloproteases, serine proteases, hyaluronidases, lipases, cysteine peptidases, C-lectins, and inhibitors, corresponded to14.5% of ESTs. Molecules involved in gene and protein expression were 44.6% of total ESTs and no-match sequences corresponded to 24.6%. Similar results were obtained by Gremski et al. (2010), who revealed that 9% of the analyzed transcripts corresponded to Phospholipase-D, comprising 20.2% of all ESTs. Thus, these authors concluded that phospholipases D, astacin-like metalloproteases, and low molecular mass insecticidal peptides comprised approximately 95% of the toxin-encoding transcripts of *Loxosceles intermedia* venom. However, there are differences between species. *L. similis*, for example, appears to exclusively have three species-related Phospholipase-D sequences (Dantas et al., 2016).

Phospholipases D

Phospholipase-D (PLD), also known as sphingomyelinase, SMase or dermonecrotic toxin, is the main responsible for dermonecrosis and the severe inflammatory response seen in loxoscelism (Forrester et al., 1978; Rodríguez e Mendéz, 2008; Chaves-Moreira et al., 2011), and are assembled in a protein family called Loxtox (Kalapothakis et al., 2007). It is the most studied toxin, due to its paramount influence on the venom biologic effect which comes from the sphingomyelin hydrolysis into choline and ceramide-1-phosphate (C1P) (Futrell, 1992; da Silva et al., 2004; Chaim et al., 2011). C1P stimulates cytosolic phospholipase-A2 leading to arachidonic acid release and formation of eicosanoids, thus promoting inflammation (Rivera et al., 2015). The removal of the choline group is capable of destabilizing cellular metabolism through several mechanisms. Plasmatic lysophosphatidilcholine, for instance, having a subtracted choline, is converted into lysophosphatidic acid (LPA), a lipid mediator able to promote numerous cellular activities, including platelet aggregation (Moolenaar, 1999). Therefore, the inflammatory response promoted by PLDs is started by interfering with cellular metabolism, regarding its growth, proliferation, and differentiation (Paludo et al., 2006; Corrêa et al., 2016). Furthermore, the main actions of loxoscelic venom are neutrophilic infiltration, complement activation, platelet aggregation, edema, increased vascular permeability, hemolysis, and renal failure (Forrester et al., 1978; Rodríguez e Mendéz, 2008; Chaves-Moreira et al., 2011).

Neutrophils' role on the inflammatory response is directly linked to the endothelial dysfunction caused by PLDs, thus leading to an indirect and dysregulated neutrophil activation, a major factor in the dermonecrosis pathophysiology (Patel et al., 1994). The deregulated leukocyte activation has been reported to cause the up-regulated expression of Interleukin-6 (IL-6), interleukin-8 (IL-8), C-X-C Motif Chemokine Ligand 1 (CXCL-1), C-X-C Motif Chemokine Ligand 2 (CXCL-2) (Dragulev et al., 2007), as well as monocyte chemoattractant protein-1 (MCP-1), contributing further to dermonecrosis, especially since IL-8 is the most potent neutrophil chemoattractant in inflammation. C1P and LPA were shown to exhibit only minor effects on the aforementioned chemokines (Rojas et al., 2017). PLDs also act as potent endothelial cell agonists, thus inducing the secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-8 and expression of E-selectin. Endothelial dysfunction is observed both on extracellular matrix

and cellular surface, leading to subendothelial vacuoles and fibrin formation, accompanied by morphologic alterations, such as cellular retraction, reduction of intercellular adhesion, and disorganization of actin filaments. There is also an imbalance in the synthesis profile of fibronectin matrix and cellular adhesion to fibronectin. These disruptions mentioned above on endothelial surfaces and cellular adhesion structures act directly on blood vessel stability and can cause leukocyte and platelet activation, increased vascular permeability, and promote disseminated intravascular coagulation (Paludo et al., 2006).

Endothelial dysfunction is also a major trigger to platelet aggregation, which, in turn, is another factor contributing to intravascular coagulation (Kurpiewski et al., 1981). PLDs are also associated with thrombocytopenia and increased blood clotting time by directly increasing activated partial thromboplastin time (APTT) and depleting coagulation factors VIII, IX, XI and XII, responsible for the intrinsic pathway (activated by contact), thus destabilizing the whole blood clotting mechanism. Direct consequences are decreased renal perfusion and systemic intravascular coagulation (Babcock et al., 1986). Platelet aggregation will also contribute to dermonecrosis, since dermal venules and arterioles will be occluded, thus contributing to hypoxia and cellular devitalization (Zanetti et al., 2002).

PLDs can also have a hemolytic action (Forrester et al., 1978) not yet elucidated, but connected to the exacerbation of the inflammatory cascade due to disruption of the phospholipidic membrane and henceforth liberation of arachidonic acid and prostaglandin E2 (PGE2) (Chalfant e Spiegel, 2005; Ribeiro et al., 2007; Chaves-Moreira et al., 2009; Chaves-Moreira et al., 2011). These hemolysins may be related to disturbances in membrane fluidity and calcium metabolism of erythrocytes. PGE2 and C1P can act in increasing intracellular calcium in erythrocytes and disturbing ionic channels responsible for maintaining cellular tonicity and osmolarity (Chaves-Moreira et al., 2011). Hemolysis can occur in a calcium-dependent manner, but also by activation of the complement system, being independent of antibody mediation (Bravo et al., 1993). Complement system is the main humoral mediator of the inflammatory process, and its activation can occur by PLDs action in activating metalloproteases, which will promote glycophorins cleavage. Glycophorins are the main membrane integral protein of erythrocytes and this cleavage will activate the alternative pathway of the complement

system, causing cellular lysis (Tambourgi et al., 2002). The classic pathway will also be activated due to exposure of the membrane phosphatidylserine layer, promoting C1q adhesion (the first factor of the complement system classic pathway), causing cellular hemolysis (Tambourgi et al., 2007).

Metalloproteases

PLDs can promote expression and secretion of metalloproteases types 2 (MMP2) and 9 (MMP9) and increase metalloprotease 7 (MMP7) expression (Corrêa et al., 2016). Metalloproteases are responsible for digesting peptides and processing extracellular matrix molecules, besides activating growth factors, interfering in platelet adhesion, increasing vascular permeability and causing, due to all that, hemorrhage. Expression of metalloproteases is paramount for cellular death and loss of keratinocyte adhesion, another contributing factor to dermatonecrosis. Henceforth, they can also aid in toxin dispersion through extracellular matrix degradation, proteic cleavage, and release of active peptides (Paludo et al., 2006; Tambourgi et al., 2010; Corrêa et al., 2016; Oliveira-Lima et al., 2016).

In *L. intermedia* venom, two metalloproteases were identified as Loxolysin A, able to degrade fibronectin and fibrinogen, and Loxolysin B, with gelatinolytic activity (Feitosa et al., 1998). The fibrinogenolytic activity was also reported in *L. reclusa* and *L. laeta* venom (Zanetti et al., 2002). Metalloproteases are highly expressed toxins on both *L. laeta* venom (8% of total transcripts) (Fernandes-Pedrosa et al., 2008) and *L. intermedia* venom (9.8%) (Gremski et al., 2010). Expression of metalloproteases may be directly connected to venom toxicity since they are the main responsible for proteolytic activity. Differences in proteolytic activity may exist even within species, as described by Medina-Santos et al. (2019), when comparing venom toxicity from both Brazilian and Peruvian *L. laeta* specimens.

Inhibitor cystine knot

Inhibitor cystine knot (ICK) or knottins are small mass peptides with a molecular mass ranging from 5 to 8 kDa, which present insecticidal activity. ICK toxins can be quite diverse in their biological activities since they can disrupt voltage-gated sodium, potassium, or calcium channels; act upon mechanosensitive channels; and nicotinic

acetylcholine receptors (Dutertre and Lewis, 2010). Four insecticidal peptides from *Loxosceles* spp. have been purified and named LiTx-1, LiTx-2, LiTx-3 (de Castro et al., 2004), and LiTx-4. They exert neurotoxic properties, since they act in ion channels and receptors in the nervous system (Matsubara et al., 2013). Venom transcriptome shows that 55.5% of all transcripts that encode toxins potentially represent insecticide peptides, with 32% related to LiTx-3, 11.4% to LiTx-2, 6.2% to LiTx-1, 3.7% to LiTx-4 and 2.3% to the neurotoxin similar to *Macrothele gigas'* Magi 3 (Fernandes-Pedrosa et al., 2008).

Translationally controlled tumor protein

Translationally controlled tumor protein (TCTP) acts within the cellular proliferation cycle, working as a calcium chelating agent, besides promoting histamine release and inducing interleukin production (MacDonald et al., 1995; Bazile et al., 2009). Boia-Ferreira et al. (2019) reported TCTP as a strong contributor to allergic and inflammatory responses in cutaneous loxoscelism, since it promotes a remarkable histaminergic effect related to pro-inflammatory properties, such as increase in microvascular permeability of skin vessels and induction of edema. TCTP is present in a relatively small amount of venom transcripts that encoded toxins, adding to 0.4% (Gremski et al., 2010).

Hyaluronidases

Hyaluronidases are molecules responsible for degrading hyaluronic acid and chondroitin sulfate. Hyaluronic acid is one of the main extracellular matrix components of vertebrates, and its degradation can increase other substances diffusion, acting in dispersing the loxoscelic venom, thus acting as venom's "spreading factor" (Menzel and Farr, 1998). *Loxosceles*-derived hyaluronidases are not able to produce necrosis on their own, but the clinical characteristics on *Loxosceles* envenoming, such as edema, erythema, and necrosis, strongly indicates extracellular matrix disturbances (Gremski et al., 2014).

Serine proteases

Serine proteases can exert a proteolytic action and are related to activating complement system (Santos et al., 2009). Transcriptome analyses have shown distinct sequence coding for serine proteases (Fernandes-Pedrosa et al., 2008; Gremski et al., 2010), and their contribution to complement system activation might play an important role in hemolysis and dermonecrosis pathophysiology, features today restricted to PLDs action (Santos et al., 2009; Gremski et al., 2014).

Serine protease inhibitors

Different groups of inhibitors of proteolytic enzymes, such as serpins, Kunitz-type, and cystatin, might be present on *Loxosceles* spp. venom (Santos et al., 2009; Gremski et al., 2014). Fernandes-Pedrosa et al. (2008) found 0.6% of the total number of ESTs analyzed in *L. laeta* to be related to enzymatic inhibitors, whereas Gremski et al. (2010) identified in *L. intermedia* venom an EST similar to serpins. The presence of such inhibitors is suggested to be related to the protection of toxin integrity, thus resisting the deleterious action of prey's proteases (Santos et al., 2009).

Antimicrobial peptides

Antimicrobial peptides (AMPs) are endogenous molecules and constitute an immune defense mechanism in several living organisms, including arachnids. In spiders, the main purpose of AMPs is to depolarize cell membranes and damage their prey tissues (Garcia et al., 2013). AMPs have shown to be promising from a biotechnological standpoint, with effects, for instance, against gram-negative bacteria (Segura-Ramírez and Silva, 2018; Ramos et al., 2019).

Venom allergens

Loxosceles venom is able to stimulate the release of large amounts of IL-8 and GM-CSF, in addition to other cytokines, thus evoking inflammatory events (Dragulev et al., 2007). Mast cells may be involved in this pathogenesis, since the administration of antagonists of histamine and serotonin are able to diminish edema and vascular permeability induced by brown spider toxins (Paludo et al., 2009). However, an immediate-type allergic reaction to spider bite remains rare; venom allergens were detected on transcriptome analyses representing 0.6% of the total sequences of *L. laeta* (Fernandes-Pedrosa et al., 2008) and 0.2% of *L. intermedia* venom (Gremski et al., 2010).

Concluding notes

Understanding the complexity of *Loxosceles* spp. venom remains a challenge for researches and health care personnel. Its several components act on basic cellular physiology, especially disturbing membrane's integrity. PLDs are highlighted as the main responsible for orchestrating systemic and dermonecrotic effects. A broader understanding of venom's composition and mechanism of action are paramount not only for treating patients with loxoscelism, but also to unravel the full venom's industrial and biotechnological potential.

CHAPTER 2

History, challenges, and perspectives on *Loxosceles* (brown spiders) antivenom production in Brazil

1. Introduction

According to the World Health Organization (WHO), antisera represent the only effective therapy for treating envenomation. Since no specific alternative treatment is available to address such ailments, antivenom production is paramount to guarantee such a valuable product. However, antivenom production - derived from horses' immunization protocols - is being neglected, since developed countries are gradually halting their production. The developing countries (the ones most affected by envenomation cases) need to optimize their production to meet their growing demand. The shortage availability of antisera has become imminent and is evolving into a global health issue. Deaths and disabilities resulting from envenomation could be avoided if enough supply of antisera (with controlled production and ensured quality) could be provided, their distribution logistics improved and clinical use better explained to the health personnel (WHO, 2006). As highlighted by Brown (2012), the investment in this type of immunotherapy has not received the same level of publicity or resolve as vaccine production or monoclonal antibody research, despite its obvious public health significance.

The true incidence of spider bite, envenomation, and lethality worldwide remain unknown and is likely under-represented due to the voluntary nature of most of the reporting processes (Nicholson and Graudins, 2003). Spider bites from specimens of the *Loxosceles* genus are responsible for the development of a syndrome named Loxoscelism, consisting mainly of dermonecrotic lesions, fever, renal failure, and hematologic disturbances (Futrell, 1992; Swanson and Vetter, 2006). These spiders, popularly known as "brown spiders", are widely distributed in the Brazilian territory and have at least 17 species cataloged in the country (Bertani et al., 2018). They are recognized as a health problem in Brazil since 1957 (Rosenfeld et al., 1957) and play the leading role of spider

envenomation in the country (Fig. 1), mainly due to its high intra-domicile infestation (Ribeiro et al., 1993).

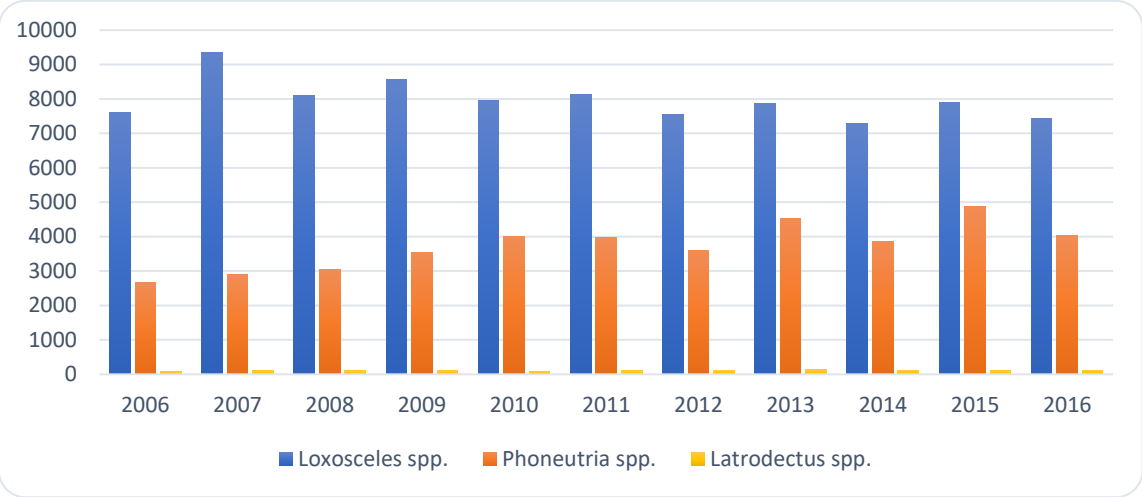

Fig. 1. Spider bites according to genus registered in Brazilian territory between 2006 and 2016. *Loxosceles* is the most relevant genus in comparison to *Phoneutria* or *Latrodectus*. Source: Ministério da Saúde/SVS - Sistema de Informação de Agravos de Notificação (Available at: http://portalsinan.saude.gov.br/dados-epidemiologicos-sinan. Accessed 01/31/2018).

Although some studies highlight beneficial treatments against brown spider bites, such as dapsone and hyperbaric oxygen (Beilman et al., 1994), tetracycline (Okamoto et al., 2017), or plasma exchange (Abraham et al., 2015), using the specific antivenom remains the primary medical resource for treating *Loxosceles* envenomation (Hogan et al., 2004; Pauli et al., 2006). However, assuring a sufficient antivenom supply with a certified quality has been proven a challenge. Some hindrances, such as cost, venom acquisition, and animal welfare issues can be pinpointed as some of the several challenges faced by the institutions responsible for antivenom production (WHO, 2006; De Roodt et al., 2007; Gutiérrez et al., 2007; Gutiérrez et al., 2009), but promising studies involving detoxification, recombinant toxins and synthetic epitopes (Kalapothakis et al., 2002; Ribeiro et al., 2007; Silveira et al., 2007; Appel et al., 2008; Mendes et al., 2013; Dias-Lopes et al., 2014) may represent a new path for optimizing antivenom procurement, for example, in innovative immunization protocols (Olvera et al., 2006; Figueiredo et al., 2014; Duarte et al., 2015). Thus, the aim of this study is to review the main aspects of *Loxosceles* antivenom production in Brazil, highlighting its history, the main challenges faced and future production prospects.

2 Loxosceles antivenom production

2.1History

The discovery of antivenom serum therapy happened in France in the XIX century; it was presented to the French Society of Biology in 1894 by representatives of the National Museum of Natural History and the Pasteur Institute, the two leading Parisian research institutions from that time. In Brazil, such production and development happened primarily due to the efforts of Vital Brazil Mineiro da Campanha (1865-1950), a pioneer in evidencing the specificity of antivenom sera (Bochner, 2016). Accompanied by Jean Vellard, several studies with arachnids were performed. In 1924, they started studying venomous spiders and, in 1936, studies around the physicochemical properties of venoms and their action on different laboratory animals were assessed. Based on these studies, *Loxosceles* genus was erroneously characterized as possessing venom of low potency to humans. In 1961, this premise was maintained by Bücherl, when he concluded that due to the small size of *Loxosceles* specimens' fangs, their lethality to humans would be unlikely. However, in that same year, technicians of the Butantan Institute found *Loxosceles* spiders on the location of spider bites' accidents, initiating an experimental antivenom production that has gained commercial levels since 1964. Improvement of different methods to gain access to specimens, better care of animals in captivity, and optimization of venom extraction techniques were paramount in enabling the study of arachnid venoms under different perspectives and their action on humans (Lucas, 2015).

In Brazil, antivenoms are liquid preparations that contain purified equine Fab'2. Since 1986, they are all manufactured by four large public laboratories, which are Centro de Produção e Pesquisa de Imunobiológicos (CPPI), Fundação Ezequiel Dias (FUNED), Instituto Butantan and Instituto Vital Brazil (Araujo et al., 2008; Ministério da Saúde do Brasil, 2017). Regarding specific loxoscelic antivenom procurement, CPPI (Brazil), National Institute of Health (Peru), and Bioclon Institute (Mexico) are highlighted (Hogan et al., 2004). According to Gutiérrez et al. (2007), Brazil holds a self-sufficient production capacity and procures a varied range of immunobiologicals. A National Program for Self-Sufficiency in Immunobiologicals was created in 1985. In addition to the long Brazilian tradition in the production, control, distribution of antivenom serum, and treatment of victims paid by the government, relies on a ubiquitous concern about collecting data of envenoming cases ever since the first antivenom ampoule was procured in 1901 (Bochner,

2013). Therefore, Brazil is one of the few countries in Latin America with a national information system for monitoring incidents involving venomous animals, created in 1986, labeled Brazilian Information System on Diseases of Compulsory Declarations ("Sistema de Informação de Agravos de Notificação", SINAN), which provides the Brazilian reality regarding any sort of envenoming (Gutiérrez et al., 2009; Bochner, 2013). This information system is responsible for epidemiological surveillance, modernization of technology and infrastructure for antivenom production, standardization of diagnostic and therapeutic conducts, a thorough analysis of the geographical distribution of venomous animals in the whole country, as well as coordination of permanent training programs for health personnel (Gutiérrez et al., 2009). However, the need to better training health personnel and promote educational programs is evident when the majority of the spider bites reported do not come accompanied by proper genus identification (Fig. 2).

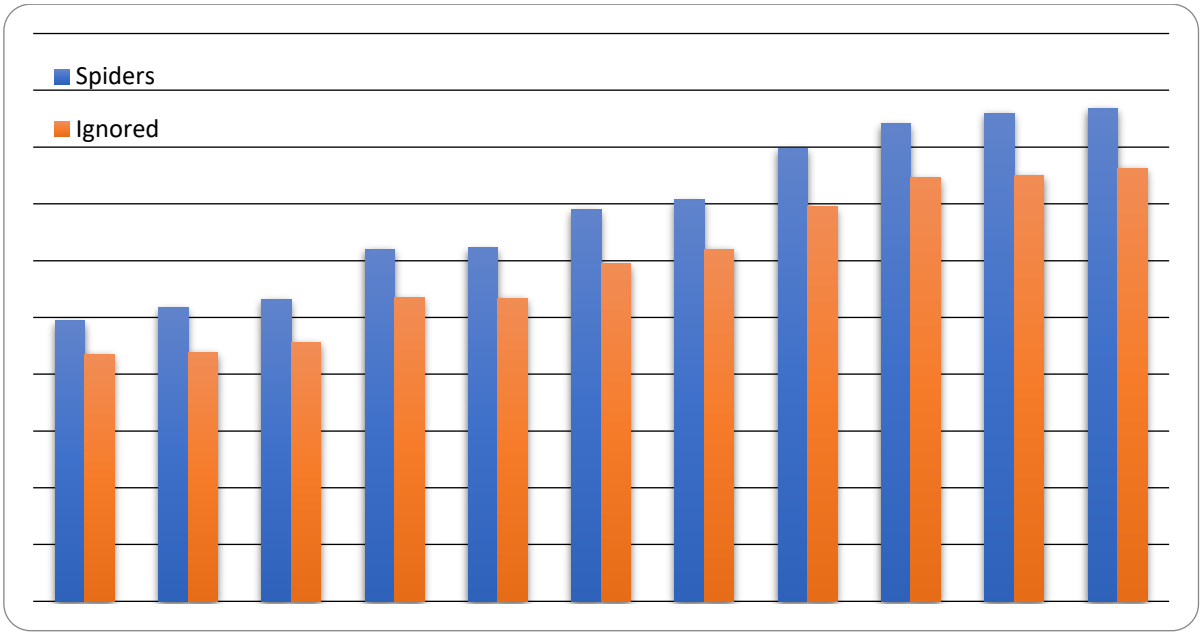

Fig. 2. Total of spider bites registered in Brazilian territory between 2006 and 2016. Note the lack of genus identification in the majority of cases reported (Ignored) over-identified spider genus (Spiders). Source: Ministério da Saúde/SVS - Sistema de Informação de Agravos de Notificação (Available at: http://portalsinan.saude.gov.br/dados-epidemiologicos-sinan. Accessed 01/31/2018).

2.2 Main challenges faced in antivenom production, distribution, and quality control

There is an ongoing reduction regarding the number of antivenom manufacturers around the world, accompanied by a contraction in the range of immunobiologicals available, especially those used for the treatment of snake and spider bites. Even developed

countries are halting its production, due to the unattractive profitability of antivenom development and production, which inevitably led to a pressure of existing manufacturers to absent oneself from such a market. However, the need for further expenditure on novelty protocols and other procedural refinements intended to improve antivenom production protocols prevails. Therefore, it is paramount that policymakers and antivenom manufacturers optimize production, as well as act in promoting global initiatives to secure the access to these immunobiologicals through partnerships between the public sector, pharmaceutical industry, and international organizations (Fry et al., 2003; WHO, 2006; Brown, 2012). According to Gutiérrez et al. (2007), there is an urgent need to develop international guidelines for the production of antivenoms. Hence, permanent regional training programs targeted at the professional and technical staff working in antivenom producing laboratories are urgently needed.

Curiously, some of the main challenges faced during the first steps of spider antivenom production remain up until today. Linking the accidents by spiders and performing a correct taxonomic identification, as well as collecting enough spiders to obtain enough amount of venom and improving the methodology for extracting a high-quality venom (Lucas, 2015) remain some of today's difficulties to be overcome. The diagnosis of envenomation is usually based on clinical presentation since the spider is hardly ever available for identification. *Loxosceles* presents yet a further aggravate, which is the late presentation of victims. The effectiveness of antivenom for the treatment of the necrotic skin lesions is, therefore, unclear (Nicholson and Graudins, 2003). Also, within the clinical context, there is a misidentification of physically similar species, which may result in selecting the wrong antivenom type. Venom composition among spiders, snakes, and scorpions can vary widely due to a natural selection or even differences in diet, which can have direct implications regarding antivenom production and its further effectiveness (Fry et al., 2003). Therefore, to optimize antivenom production, it is necessary to understand which medically important species for each region are, concentrating the efforts of antivenom production on species of considerable public health importance. That can only be achieved by extensive epidemiological studies focused on community surveys and pharmacological characterization of venoms. Also, there should be well-established systematics in identifying the relevant venomous species, which should aid in collecting specimens with epidemiological relevance to produce antivenoms (Fry et al., 2003; Gutiérrez et al. 2007). Guimarães et al. (2013), that studied the antivenom efficacy

for both Peruvian and Brazilian *Loxosceles laeta*, concluded that antivenoms produced in Brazil were able to act upon the Peruvian venom, which could facilitate an antivenom partnership for antivenom supply between the two countries.

Carmo et al. (2015) highlight that significant improvements were made in all phases of antivenom production, such as the advancements in immunization protocols and antibody preparations, groundbreaking immunoglobin purification processing, and constant updates of quality control measures aiming to ensure the utmost safety for the user. Redwan (2009) asserts that there are two main problems of animal-derived therapeutics: their immunogenicity induction and issues regarding their biosafety. Since side-effects can range from rashes to severe adverse reactions (even death), anaphylaxis, anaphylactoid reactions, and serum sickness need to be predicted and handled properly. Once more, actions aiming for health personnel education are imperative.

According to Gutiérrez et al. (2009), maintenance of a cold chain is an obstacle faced by many tropical countries in antivenom distribution and use, since liquid preparations should be maintained between 5±3°C, having three years before expiration. On the other hand, freeze-dried preparations might extend the immunobiological shelf-life in over five years. Silva and Tavares (2012) compared to powder and liquid bothropic antivenoms and concluded that there was no statistical difference in the number of adverse reactions promoted using both antivenoms, providing one more step toward the use of powdered antivenoms in regions where the cold chain cannot be maintained.

Gutiérrez et al. (2007) also highlight difficulties regarding handling and proper care of animals used in immunization, plasma fractioning, ultrafiltration, maintenance of aseptic filling, lyophilization, cleaning, and sanitization of equipment. These authors also propose applying the 3R concept to the antivenom production: *Replacement*, *Reduction*, and *Refinement*. Replacement would be illustrated by employing immunochemical methods and establishing *in vitro* functional methods that correlate with *in vivo* toxic activities. Reduction would concentrate on using the minimum number of animals possible and collecting a maximum of information from them. Refinement would mainly consist of reducing the length of essays' time to minimize animal suffering, as well as promoting animal welfare initiatives, such as analgesia and reduction of stress. Nevertheless, although new techniques are being implemented to avoid animal suffering, such as the *in vitro* toxicity testing and the *in vitro* antivenom efficacy testing (Ramada

et al., 2013), some hindrances are faced. For example, the overall toxicity of any venom relies on its interaction with tissues, with complex pharmacokinetics and dynamic interactions, which cannot be assessed or predicted by *in vitro* evaluations (Fry et al., 2003).

Quality control of immunobiologicals aims to ensure that the intermediate and the final products conform with physical-chemical and biological characteristics described in guidelines and pharmacopeia monographs. To ensure that the product meets the safety and efficacy requirements for human use, some essential aspects must be considered, such as licensing (accompanied by well-conducted preclinical and clinical trials), good manufacturing practices and quality control *per se* (Araujo et al., 2008). Trying to address these needs, efforts have been continuously made regarding the development of quality control laboratories, both by manufacturers and national regulatory agencies, as an attempt to assure that antivenoms fulfill quality and safety requirements established for these immunobiologicals (Gutiérrez et al., 2007). In 1984, the CYTED (*Ciencia y Tecnología para el Desarollo*) was signed by 21 Portuguese- and Spanish-speaking countries aiming for an Ibero-American strengthening of the public institutions in charge of the production and quality control of antivenoms (Gutiérrez et al., 2009). A partnership involving the WHO, the Pan American Health Organization (PAHO), and several Latin American Ministries of Health was proposed to promote awareness of envenomation impact, provide support to the technical capacity of local antivenom producers and expertise of regulatory authorities. In Brazil, since 1987, the National Control Laboratory of the Brazilian Ministry of Health has been testing all lots of antivenoms for their sterility, volume, pH, and presence of pyrogens, phenols, sodium chloride, proteins, protein nitrogen, and ammonium sulfate. No potency test is performed for loxoscelic antivenoms due to the lack of reference for such (only bothropic and crotalic antivenoms are evaluated in this aspect). A survey made by Araujo et al. (2008) showed that between 2000 and 2006, 134,650 ampoules of polyvalent arachidic antivenom and 12,618 ampoules of loxoscelic antivenom were evaluated. Within a total of 2,513,690 ampoules tested (including snakes, spiders, scorpions, and caterpillars antivenoms), only 1.29% of the lots were considered of unsatisfactory quality. These authors then concluded that the small incidence of rejected lots indicates that the manufacturers met the proper requirements for lot release, providing high-quality immunobiologicals for the Brazilian Public Health System.

2.3 Prospects in antivenom production

According to Gutiérrez et al. (2007), collaborative projects should be made aiming the more detailed characterization of the biochemical, toxicological and immunological characteristics of the most relevant venoms for each region, accompanied by a preclinical spectrum of efficacy of antivenoms produced, better knowledge on antivenom clinical safety and efficacy through controlled and proper designed clinical trials, and introduction of *in vitro* techniques in substitution for procedures using animals. All these steps are crucial to produce effective antivenoms.

New techniques in antivenom production must be feasible, cost-competitive, and yield more efficacious and safer antivenoms. According to Laustsen et al. (2016), over 10 million spider toxins and the pool of structural and bioinformatics data are an outstanding foundation to the development of modern antivenoms. The accumulated data on the structures and toxicities of venoms are an excellent information source for the development of next-generation antivenoms, massively relying on biotechnological approaches and modern drug discovery techniques. Some biotechnological techniques for the generation, isolation, and production of monoclonal antibodies or antibody fragments have recently been applied in the development of experimental antivenoms against spider bites.

The advent of recombinant DNA technology made possible the development of heterologous protein expression techniques, which provide the basis for alternative methods in antivenom production. Some neutralizing anti-loxoscelic sera have been obtained using different sphingomyelinases isoforms. Araujo et al. (2003) immunized animals using a recombinant SMase D protein and the produced serum neutralized dermonecrotic activity and lethality in rabbits and mice. De Almeida et al. (2008) immunized horses with a mixture of three recombinant SMase D isoforms, and they also produced a neutralizing anti-loxoscelic serum. This serum showed similar or better neutralizing capacity when compared to anti-arachnid commercial serum (De Almeida et al., 2008). Despite the promising results, these recombinant proteins kept their enzymatic activity and, therefore, they showed toxic effects in the immunized animals. To overcome those issues, a formulation containing only multiepitopes previously validated as chimeric proteins (synthetic or recombinant) could be used as antigen to immunize animals. In this way, Mendes et al. (2013) constructed and expressed a chimeric protein

(rCpLi), containing three epitopes from the LiD1 sequence, by molecular biology. The protein did not show toxicity and it was recognized by the sera produced against chimera epitopes by ELISA and immunoblotting. rCpLi was used to immunize rabbits and it stimulated a humoral response. The produced antibodies individually recognized each one of the epitopes which were contained in the chimera. Furthermore, anti-rCpLi antibodies pre-incubated withrLiD1neutralized dermonecrotic and hemorrhagic activities of the protein similar to the neutralization by antibodies developed against the total protein. Considering the success of this strategy, Figueiredo et al. (2014) used the same chimeric protein (rCpLi) to immunize horses, using it alone or combining it with initial doses of crude venom. Such approaches were compared with the traditional antivenom preparation methodology, which uses a mixture of *L. intermedia*, *L. gaucho,* and *L. laeta* crude venoms. The immunization protocol composed by initial doses of venom, followed by doses of rCpLi, generated antibodies with the same reactivity detected by traditional antivenom ELISA against *L. intermedia* venom. *In vivo* studies showed that this serum neutralized the loxoscelic venom demonecrotic activity and some immunized animals produced suitable sera for therapeutic use since their potency meets the established criteria for the production of immunobiological. Besides, it is worth to mention that the amount of crude venom required for sera production was reduced by 67%.

Regarding the improvement of antibodies neutralizing potential and to mimic the loxoscelic crude venom, Lima et al. (2018) included epitopes of other toxins belonging to different molecular classes in the rCpLi, producing another chimera, called rMEPLox. *In vivo* and *in vitro* neutralization assays showed that anti-rMEPLox antibodies could efficiently neutralize the activity of sphingomyelinase, hyaluronidase, and metalloproteinase from *L. intermedia* venom. Obtained data provided insights for the use of this multiepitopic protein as a suitable candidate for experimental vaccination approaches or for antivenom production against *Loxosceles* spp.

Stephano et al. (2005) highlight that the main biological phenomena involved in the production of hyperimmune sera are connected to the adequate specific antibody titer and the chemical and physical procedures used for processing plasma. Based on these premises, recombinant toxins and synthetic peptides can be applied in novelty immunization protocols since they do not radically change the nature of antivenoms and exclude the need for laborious venom extraction. These factors enable a less hazardous

production process, remove the effect of individual venom variability, produce a safer antivenom, and yield better antibody titers since the only medically relevant epitopes are used for immunization. Generation of recombinant proteins and antibody fragments using phage display technology could bring several benefits, especially when genetic engineering techniques act upon modifying the antibodies and their fragments to provide a better affinity maturation (Laustsen et al., 2016). Cloning and synthetically production of antibodies may lead to the optimization of antibody usage, turned into optimized pharmacokinetic properties and enhanced shelf-lives. A study conducted by Hmila et al. (2010) with scorpion venom, showed that nanobodies, a single variable domain of a heavy-chain antibody, could reach to toxins already distributed to organs and other deep tissues - especially due to its low molecular weight - thus ameliorating the action of horse-derived antivenom.

De Roodt et al. (2007) propose that the inclusion of *Loxosceles* venoms, or their components with different characteristics in the immunogenic mixtures for antivenom production, which might result in a good polyspecific *Loxosceles* antivenom with better neutralization of the different *Loxosceles* venoms of epidemiological significance. These authors tested the neutralization of specific *Loxosceles* antivenoms and heterologous antivenoms and concluded that despite better neutralization capacity of specific antivenoms, cross-reactivity is present and can be very useful in polyspecific antivenom production, corroborating with Duarte et al. (2015), while evaluation anti-loxoscelic horse serum produced against a dermonecrotic protein. The serum was able to neutralize the lethal effect of *Loxosceles laeta*, despite being produced with a *Loxosceles intermedia* recombinant protein.

Tambourgi et al. (2010) state that a lack of deep understanding regarding the venom complex action mechanism has been preventing the development of new and effective therapies. Stephano et al. (2005) highlight the importance of deep immunology knowledge to aid in optimizing immunization schedules in horses used to produce antivenoms. Protocols with lower venom concentrations extending the dose intervals can promote a higher antibody levels since these conditions seem to guarantee an efficient memory induction and the avoidance of clonal deletion or anergy to relevant epitopes. Adjuvants also need to be considered, since they can have clinical consequences and

contribute to animal welfare issues involving antivenom production animals (Mota et al., 2006).

Despite the obvious scientific advances in the immunological and technical areas of antivenom production, Carmo et al. (2015) state that there is an existing gap between academic research and antivenom producers, which needs to be narrowed.

3.Concluding notes

Loxoscelism is a public health issue in Brazil, and antivenom remains its sole effective treatment. Despite its long history, antivenom obtainment faces continuing challenges, such as its inherent laborious processing, difficulties in harvesting venom from a sufficient and representative number of specimens, costs, biosafety, logistics of distribution, and medical knowledge in establishing the proper treatment. Brazil has a self-sufficient antivenom production, but still suffers from problems regarding logistics and maintenance of a cold chain during distribution, as well as insufficient training of health personnel. Despite the gloom context in which loxoscelic antivenom production withstands, new perspectives are arising, ranging from novelty immunization protocols supported by biotechnological tools to a growing concern for animal welfare and quality control issues.

CHAPTER 3

Clinical effects of immunization protocol using *Loxosceles* venom in naïve horses

Abstract

Loxoscelism is an envenomation syndrome caused by spider bite from *Loxosceles* genus. Antivenom is the only effective therapy against such ailment. Antivenom is produced by immunizing horses and the Center of Production and Research of Immunobiologicals (CPPI) is one of the few centers in which specific loxoscelic antivenom is produced. Eleven healthy horses, never immunized, were evaluated on three different periods: T0 (before immunization); T1 after their first loxoscelic antivenom immunization; and T2 after their first commercial bleeding. Horses were clinically evaluated, sampled for blood, and underwent electrocardiographic (ECG) recordings. Significant clinical findings were the several suppurated subcutaneous abscesses due to the use of Freund's adjuvants and thrombophlebitis due to systematic venipunctures for commercial bleeding procedures. Horses at T2 presented the most blood alterations including reduced packed cell volume (PCV), red blood cells (RBC), and hemoglobin. Liver and renal functions were unaffected. Hyperproteinemia occurred due to increase in globulin levels. ECG showed arrhythmias in few horses in T2, such as second-degree atrioventricular block (AV-block), as well as an increase in T and R waves. In general, horses were found healthy during the entire cycle of antivenom procurement, however, needing of more close observation after the bleeding procedures for commercial antivenom procurement.

1. Introduction

Spider bites of the genus *Loxosceles* have been associated with clinical manifestations such as dermonecrotic lesions with gravitational spreading and systemic toxicity, including hematological disturbances and acute renal failure (Swanson and Vetter, 2006; Silva et al., 2009; Chaim et al., 2011). Antivenom remains the only effective treatment against this envenomation (Pauli et al., 2006; WHO, 2006), and according to Gutiérrez et al. (2007), Brazil holds a self-sufficient production capacity and procures a varied range of immunobiological. The Center of Production and Research of Immunobiologicals (CPPI), housed in Paraná (PR) state, Brazil, is one of the few Latin American factories that produce loxoscelic antivenom (Hogan et al., 2004).

Despite its obvious public health importance, the demand for spider antivenom faces numerous challenges. Chaim et al. (2011) highlight that there are several difficulties to be overcome regarding spider venom manipulation, such as bureaucracy in capturing

specimens for venom extraction and low volume of venom obtained per extraction. Horses are the most used species for antivenom production around the world. Duarte et al. (2015) emphasize that one of the hindrances faced by antivenom producers is directly linked to horses' welfare when submitted to immunization protocols. It is important to stress that crude venoms used in the immunization protocols do not undergo any detoxification process, thus assuring that no structural or epitopic modification occurs that can alter its immunogenic capacity (Angulo et al., 1997).

Little is known, however, about the consequences of systemic envenomation on antivenom-producing horses, not only regarding the venom deleterious effects (such as hemolysis), but the systematic use of adjuvants and bleedings that horses must undergo in order to procure loxoscelic antivenom. Therefore, the aim of this study is to evaluate horses' health as they partake in their first immunization protocol for *Loxosceles* spp. antivenom production.

2. Materials and methods

2.1 Ethical statement

All procedures involving horses were conducted according to animal welfare guidelines after approval by the Ethical Committee for the Use of Animals of the Federal University of Minas Gerais (CEUA/UFMG), under protocol number 159/2019 (Annex I).

2.2 Horses

Eleven healthy crossbred horses, both male (8) and female (3), weighting 498.64±72.62 Kg and of 6.7±1.5 years of age were used. Horses belonged to and were housed on CPPI, Piraquara, PR, Brazil. Animals were kept in pasture and had access to 6 Kg of alfalfa hay, 2 Kg of a 12% protein commercial equine ration, plus 2 Kg of hydrated/germinated oat grains per day. Mineral salt and freshwater were also provided *ad libitum*. Before engaging in immunization protocols, horses were vaccinated against tetanic toxoid, leptospirosis, strangles, influenza, rabies, and encephalomyelitis, as well as dewormed. All horses had not hitherto partaken in any immunological protocol or experiment and were acquired from certified properties free of glanders and Infectious Equine Anemia.

2.3 Venom and immunization protocol

Venom was obtained from spiders captured within Paraná and Santa Catarina states, Brazil, and kept under controlled conditions in CPPI. Specimens of *L. intermedia*, *L. gaucho*, and *L. laeta* had their venom extracted after being restrained from feeding for 30 days and undergoing an electrical stimulus of 12 V applied on the cephalothorax region. The venom pool obtained was dehydrated and kept on -20°C, in the dark, until its use. Electrophoretic profile of CPPI *L. intermedia* venom is illustrated on Fig. 3.

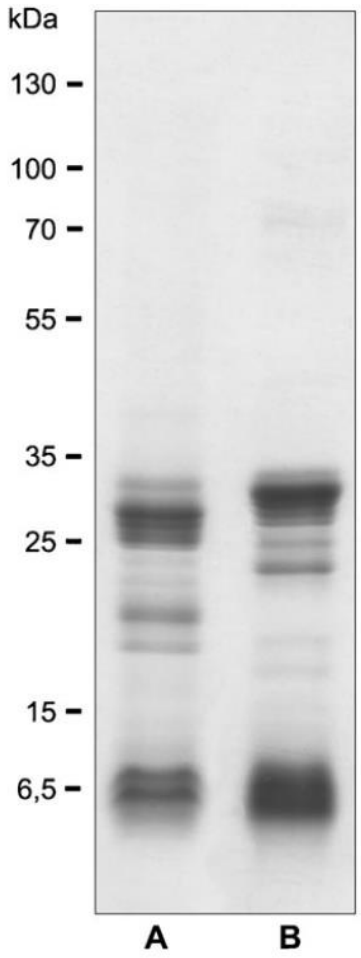

Figure 3. SDS-polyacrylamide gel electrophoretic separation of *L. intermedia* venom harvested at CPPI. (A) A non-reduced sample and (B) a reduced sample. Numbers on the left correspond to the positions of molecular weight markers (in kDa). Image gently provided by CPPI.

Horses followed an immunization schedule shown in Table 1, reaching an industrial antivenom procurement level after 221 days. Horses were clinically examined, underwent electrocardiographic recording (ECG) and had their blood sampled on three different moments: T0 (before day 1 of the cycle), T1 (day 115; after their first hyperimmunization and reimmunization); and T2 (day 225; four days after the first official bleeding for antivenom procurement). Sampling for Biological Quality Control took place on days 38,

113 e 207 of the immunization cycle. This sampling was taken by blood withdraw from the jugular vein into a clot activator tube and was immediately sent for analyses regarding its overall quality and antibody titration. After an adequate antibody titration, it was then performed the bleeding for antivenom industrial procurement, in which 7 L of blood were sampled three times every other day, adding to 21 L of total volume. Bleeding for industrial antivenom procurement took place on days 214, 216, and 221 of the cycle.

Table 1. Immunization protocol of naïve horses using *Loxosceles* spp. venom

Immunization status	Day of the cycle	Total Venom amount	Venom amount per species of *L. intermedia*, *L. laeta* and *L. gaucho*	Saline Amount (0.85%NaCl)	Adjuvant
T0	0	Clinical examination, blood sampling, ECG recordings			
Hyperimmunization	1	300 µg	100µg	1200µL	1500µL of Complete Freund's Adjuvant
Hyperimmunization	11	450 µg	150µg	1050µL	1500µL of Incomplete Freund's Adjuvant
Hyperimmunization	22	750 µg	250µg	2250µL	No
Hyperimmunization	31	750 µg	250µg	2250µL	No
Sampling for Biological Quality Control	38				
54-day rest					
Reimmunization	92	5 mg	1.67mg	No	5 mL of Incomplete Freund's Adjuvant
Reimmunization	106	5 mg	1.67mg	5 mL	No
Reimmunization	113	5 mg	1.67mg	5 mL	No
Sampling for Biological Quality Control	113				
T1	115	Clinical examination, blood sampling, ECG recordings			
74-day rest					
Reimmunization	187	5mg	1.67mg	No	5 mL of Incomplete Freund's Adjuvant
Reimmunization	201	5 mg	1.67mg	5 mL	No
Reimmunization	207	5 mg	1.67mg	5 mL	No
Sampling for Biological Quality Control	207				
Bleeding for antivenom industrial procurement	214 216 221				
T2	225	Clinical examination, blood sampling, ECG recordings			
		Antivenom industrial procurement			

2.4 Clinical and hematological evaluation

Horses were restrained using a halter and examined inside a stock. No sedation was needed. Horses underwent a thorough physical examination, encompassing inspection, measurement of parametric indexes, and clinical evaluation of biological systems, according to Speirs (1992). A form was previously designed and fulfilled accordingly (Annex II)

Blood sampling was performed after the antisepsis of the external jugular vein region using 70% alcohol. Ethylenediaminetetraacetic acid (EDTA) and clot activator tubes were used (*BD Vacutainer*® - *Becton Dickinson*) in order to perform hematologic and biochemical analyses, respectively. Hematimetric indexes were obtained using an automatic cell counter (*pocH-100Iv-Diff-Sysmex*®) with a software calibrated for the equine species; serum biochemistry was analyzed by an automatized biochemistry equipment (*Cobas Mira Plus*®) under a clinical pathologist supervision. Blood parameters evaluated were as follows: red blood cell count (RBC); hemoglobin; packed cell volume (PCV); white blood cell count (WBC); red blood cell distribution width (RDW); lymphocytes, and sum of other WBC, such as neutrophils, monocytes and basophils (OTH); total platelet count (PLT); mean platelet volume (MPV); platelet distribution width (PDW); platelet clump (P-LCR); urea; creatinine; alanine aminotransferase (ALT); aspartate transaminase (AST); alkaline phosphatase (ALP); gamma-glutamyl transpeptidase (GGT); glucose; amylase; total proteins (TP); albumin; globulins; cholesterol; triglyceride; lactate; and lactate dehydrogenase (LDH).

2.5 Electrocardiographic evaluation

Horses underwent an ECG evaluation using a portable 12-channel digital electrocardiograph (*TEB ECG Vet*® - *Tecnologia Eletrônica Brasileira S.A.).* ECG recordings were acquired in a quiet environment and with horses in an orthostatic position. Electrodes were fixed using alligator metal clips embedded in alcohol and attached to the horses' skin using Dubois configuration (Costa et al., 2017), in which electrodes 1 and 2 are placed next to the spine tuberosity of both left and right scapula, electrode 3 on the xiphoid process of the sternum and electrode 4 on the proximal cranial

region of the left forelimb. Recordings were made at 25mm/sec speed and sensitivity of 1 cm = 1mV. Bipolar (DI, DII, DIII) and augmented unipolar (aVR, aVL, aVF) leads were recorded. The following parameters were evaluated: cardiac rhythm; cardiac frequency; P (ms); P (mV); PR, QRS and QT intervals; R and T waves and ST segment levels. The electric axis was determined by QRS polarity in the bipolar and unipolar augmented leads.

2.6 Statistical analysis

Statistical analysis was carried out using the SAS (version 9.0) software program. The obtained data were statistically analyzed using a mixed linear model approach of SAS (PROC MIXED), using first-order autocorrelation covariate structure. Animals were considered as a random factor with repeated measurements over time. P values < 0.05 were considered to indicate significance.

3 Results and discussion

3.1 Clinical examination

Animals were alert during all three evaluation periods, thus presenting no alteration of mental status. The major alteration observed during the inspection was, undoubtedly, the appearance of subcutaneous abscesses. No abscesses were diagnosed in T0, whereas multiple were diagnosed in T1, and on T2 they presented a suppurative characteristic. According to the immunization protocol described in Table 1, it is noticeable the use of Freund complete or incomplete adjuvant during the hyperimmunization cycle and as starters on the reimmunization cycles. According to Apostólico et al. (2016), adjuvants are essential for enhancing and directing adaptative immune response, such as the one observed in immunization protocols aiming antivenom procurement. These authors conceptualize adjuvants as substances that increase immunogenicity due to their ability to stimulate a strong humoral and cell-mediated immunity. In 1924, Gaston Ramon (Pasteur Institute) described a higher specific antibody titer in horses with cutaneous abscesses, developed at the injection site during immunization. It was then first described that substances capable of inducing inflammation could improve antisera production. These inflammatory effects could also be seen in the present study by the general increase

of lymph nodes between the evaluation periods, achieving its maximum lymphadenomegaly at T2.

In the 1930s, Freund adjuvant was formulated, based on a water-in-mineral oil emulsion that contained heat-killed mycobacteria, such as *Mycobacterium tuberculosis* (Opie, 1937). Freund's complete adjuvant has mycobacteria in its composition, functioning as a guide in making T-lymphocytes acquire a Th1 pattern and acting as a mediator for promoting delayed-type hypersensitivity (Apostólico et al., 2016). However, its use is severely regulated by animal welfare guidelines due to its reactogenic characteristics, inducing granulomas, abscesses and ulcerative necrosis at the injection site (Billiau and Mathys, 2001), such as the ones observed on the present study. Freund's complete adjuvant is used only once in horses used for antivenom procurement since the hyperimmunzation cycle is only imposed to naïve horses. Freund's incomplete adjuvant, however, is used during the hyperimmunization cycle and the subsequent reimmunization cycles. Differently from Freund's complete adjuvant, Freund's incomplete adjuvant does not have mycobacteria in its composition but acts as a means of continuous antigen release from its oily deposit, increasing antigen lifetime, enhancing phagocytosis, leukocyte infiltration and cytokine production (Mussener et al., 1995). These characteristics are paramount for an efficient adjuvant since they are employed aiming to decrease the amount of antigen used, reduce the number of doses required to induce immunity, and increase the seroconversion rate (Apostólico et al., 2016). Calabria et al. (2019), while using a hybrid recombinant spider toxin to produce loxoscelic antiserum, concluded that their works' highlight was the prospect of decreasing the number of antigens received by animals used in immunization protocols for antisera production, besides ameliorating the problem of limited amount of crude venom, time-consuming venom extractions and animal handling. In the present study, horses took approximately 221 days to achieve a desired commercial seroconversion, a satisfactory period according to CPPI data and history. It would be beneficial for the production scale; however, if such a period could be shortened and done with a smaller venom amount, as aforementioned by Calabria et al. (2019).

Results regarding body weight, pulse and respiratory rates, and rectal temperature are described in Table 2. No statistical difference was observed regarding these parameters comparing the three evaluation periods.

Table 2. Body weight, pulse and respiratory rates, and rectal temperature of horses that underwent immunization with *Loxosceles* spp. venom examined in three different evaluation periods (mean ± SEM)

Clinical parameter	Evaluation period		
	T0	T1	T2
Body weight (Kg)	509.0±14.0	498.6±23.0	507.4±13.70
Pulse rate (ppm)	51.5±3.37	49.4±2.84	53.7±3.80
Respiratory rate (mpm)	20.8±1.77	22.3±1.69	19.0±4.97
Rectal temperature (°C)	38.0±0.25	38.3±0.07	37.8±0.15

No statistical difference observed ($p>0.05$, mixed linear model)

It is well known that immunization protocols have a catabolic effect on horses' metabolism since high amounts of energy and protein mobilizations are made in order to produce globulins (Waghmare et al., 2014). The absence of weight difference between the evaluation periods proves that nutritional management is correctly conducted within CPPI, being a major factor that addresses animal welfare regulations.

Rectal temperature also had no statistical differences between the evaluation periods, and values were within the normal range for the equine species established by Speirs (1992) (37.5-38.3 ºC). However, it is important to highlight that data collection was punctual, and that animals probably had experienced fever during their immunization period due to inflammation provoked by adjuvants, that probably took place after days 11, 92, and 187, when horses did not undergo a clinical examination.

No statistical difference was observed in pulse and respiratory frequencies, two major parametric indexes that are directly connected to stress but can also work as compensation tools used by the body in caloric stress and anemia (Speirs, 1992; Malikides et al., 2001). Despite the lack of statistical difference, it is visible an increase in the standard error in T2, which can be linked to an individual variability observed on this evaluation period, which requires a more comprehensive interpretation that must be linked to other clinical parameters, such as mucous membranes. All horses presented mucous membranes with standard coloration (healthy pink and moist) during the evaluation periods, except on T2, in which mucous membranes of six animals (54.6%) were pale. T2 was an evaluation period that took place after the industrial bleeding phase, in which during a six-day period horses were deprived of approximately 21 L of blood. Malikides et al. (2001), while evaluating horses that have undergone similar bleeding procedures, concluded that major alterations were observed within the first 24 hours after blood withdraw, which could

explain the lack of statistical difference on the present study since samples were taken 96 hours after bleedings took place, which might also explain the lack of alterations regarding capillary refill time (CRT). CRT remained inferior to two seconds (within the normal range for the equine species), thus proving that animals showed no signs of dehydration or major cardiovascular compensations detected by the clinical examination. ECG, however, was able to detect what might be cardiac compensations (item 3.3 further discussed in this section).

One relevant cardiovascular pathology clinically diagnosed, however, was thrombophlebitis of the external jugular vein, a common clinical complication on equine internal medicine. Thrombophlebitis is defined by a vein thrombosis with concurrent inflammation. Most of this complication arise from venous catheterization, and the external jugular vein is the most affected site, due mainly to its caliber, length and accessibility (Barakzai and Chandler, 2003; Divers, 2003; Milne and Bradbury, 2009). In 1856, Virchow had already established the triad responsible for thrombophlebitis' etiopathology: blood stasis, hypercoagulability, and endothelial injury. Antisepsis prior to venipuncture is paramount in order to avert thrombophlebitis (Lankveld et al., 2001; Barakzai and Chandler, 2003; Geragthy et al., 2009). However, other factors can also influence this pathology, such as the patient's clinical condition. Horses with colic or that have undergone surgical procedures, present diarrhea, endotoxemia or fever, or any other situation that can increase blood coagulability can predispose to thrombophlebitis (Lankveld et al., 2001; Divers, 2003; Dias and Neto, 2013; Dias et al., 2014). In the present study, thrombophlebitis probably developed due to the systematic venipuncture needed for the large amounts of blood withdrawal required for antivenom production.

3.2 Blood parameters

Complete blood count (CBC) results are shown in Table 3. Leukocytosis was observed on both T1 and T2 since WBC was above the reference values for the species. Statistical difference was found between T0 and the remaining evaluation periods, probably indicating an inflammatory response due to the use of adjuvants and the immunization protocol itself. The inflammatory response is corroborated by the relation between lymphocytes and OTHR. Horses in T0 presented an adequate proportion of 2 lymphocytes: 1 OTHR, whereas in T1 and T2, this proportion was noticeably altered, probably due to neutrophilia, corroborating with Angulo et al. (1997) and Netto et al.

(2004) findings. This finding was especially observed in T1, a cycle after the hyperimmunization protocol, in which Freund's complete adjuvant is employed. As previously discussed in item 3.1 of this section, adjuvants are intended to promote an inflammatory response in order to optimize humoral and cellular immunity, thus increasing antibody titers (Apostólico et al., 2016). The use of Freund's complete adjuvant in the present study was able to induce ulcers and granulomas, an effect previously reported (Billiau and Mathys, 2001), which can also explain the neutrophilia observed. Therefore, neutrophilia can come not only from a general inflammatory response but also from local inflammatory targets, such as the ones observed subcutaneously at injection sites in the present study, which add to neutrophil recruitment (Mussener et al., 1998). Similar results were observed by Angulo et al. (1997) while evaluating horses partaking in snake antivenom production using Freund's adjuvant and Waghmare et al. (2014), who observed local reactions while evaluating the use of montanide as an adjuvant. Klimka et al. (2015) conducted a work comparing both adjuvants mentioned above and its clinical effects. Montanide induced a slightly delayed increase of antigen-specific antibody titers and produced significantly lower IgG titers. Montanide was not employed on the present study immunization protocol since a delay in achieving antibody titers is detrimental to commercial antivenom procurement. Regarding clinical effects, Waghmare et al. (2014) described that horses showed less pain and physical discomfort with montanide, which is a major benefit regarding animal welfare conducts.

Table 3. Complete blood count panel of horses that underwent immunization with *Loxosceles* spp. venom in three different evaluation periods (mean ± SEM).

Parameter	Evaluation period			Reference values for horses
	T0	T1	T2	
WBC (cell x 10^3/μL)	11.4±0.93 b	17.1±1.19 a	15.1±1.24 a	5.20-13.9[1]
RBC (cell x 10^6/μL)	8.67±0.48 a	6.77±0.30 b	4.67±0.51 c	6.4-10.0[1]
Hemoglobin (g/dL)	13.3±0.83 a	10.3±0.54 b	7.30±0.95 c	11.0-17.0[1]
PCV (%)	39.8±2.49 a	31.4±1.78 b	21.91±1.62 c	32.0-47.0[1]
PLT (cell x 10^3/μL)	114.2±17.4 b	179.5±15.3 a	98.3±23.6 b	120.0-256.0[1]
Lymphocytes (%)	33.5±2.89 a	18.7±1.58 c	28.8±2.04 b	
OTHR (%)	66.5±2.89 b	81.3±1.58 a	61.1±6.49 b	
Eosinophils (%)	0.00±0.00	0.00±0.00	0.00±0.00	
Lymphocytes (cell x 10^3/μL)	3.68±0.31 a	3.13±0.30 b	4.19±0.31 a	1.5-7.7[1]
OTHR (cell x 10^3/μL)	7.75±0.88	14.0±1.08	10.9±1.10	
Eosinophils (cell x 10^3/μL)	0.00±0.00	0.00±0.00	0.00±0.00	
RDW-SD (fL)	38.0±0.61	39.1±0.93	39.6±0.91	
RDW-CV (%)	20.9±0.28 b	21.7±0.34 a	21.8±0.42 a	21.0-25.0[1]
PDW (fL)	9.78±0.09 a	8.83±0.17 b	7.29±0.35 c	
MPV (fL)	8.23±0.12 a	7.86±0.10 b	6.60±0.12 c	5.3 – 7.8[2]
P-LCR (%)	6.70±1.43 a	4.57±0.56 b	0.60±0.32 c	

Red blood cell count (RBC); packed cell volume (PCV); white blood cell count (WBC); red blood cell distribution width (RDW); sum of other WBC, such as neutrophils, monocytes and basophils (OTHR); total platelet count (PLT); platelet distribution width (PDW); mean platelet volume (MPV); platelet clump (P-LCR).
[1]Reference values according to Grondin and Dewitt (2010)
[2]Reference values according to Laboratory of Clinical Analysis, Veterinary Hospital, Cardenal Herrera University
a,b,c: Different letters between evaluation periods show significant difference ($p<0.05$, mixed linear model)

T2 was the evaluation period in which horses presented the most noticeable alterations regarding CBC, due to the effects of the bleeding procedures that horses have undergone before this sample collection. Pale mucous membranes, for instance, were diagnosed in 54.6% of horses during T2. This clinical finding is often linked to low RBC (Speirs, 1992). Statistical difference was observed between all evaluation periods, with lower values at T2. Horses at T2 showed PCV, RBC, and hemoglobin below the reference values for the species and hence, were diagnosed with anemia. Similar findings were described by Angulo et al. (1997) and Netto et al. (2004). Two horses kept PCV under 15% during T2, which shows a clinical concern since in most of these cases a blood transfusion is required (Mudge, 2014). Malikides et al. (2001) evaluated clinical effects on horses' physiology after severe bleedings have happened (20 mL/kg). Most of the relevant findings were described within the first 24 hours after the bleedings have happened, and horses were able to keep PCV and RBC within reference values after this

period. In the present study, however, horses underwent a systematic bleeding procedure, consisting of a 7 L/day blood withdrawal every other day for three days, which adds up to 21L of blood withdrawal and surpasses a 20 mL/kg withdrawal. The higher severity of the bleeding procedures on the present study might explain why horses kept several hematimetric indices under reference values for the species. Physiologic compensations, nonetheless, could explain why no statistical difference occurred regarding clinical findings. Severe bleeding causes reduced arterial and capillary pressure, inciting a sympathetic response. Baroreceptors and chemoreceptors induce the release of catecholamines by the adrenal medulla, thus increasing heart rate and contractility, generalized arteriolar/venous constriction, and therefore maintenance of cardiac frequency, blood pressure and respiratory rate (Guyton and Hall, 2015). This might explain why no significant alterations were reported on clinical findings in T2.

PLT showed no difference ($p>0.05$) between T0 and T2. However, T2 PLT values appeared to be inferior to reference values for the species, which is in accordance with the bleeding animals have previously undergone in this evaluation period. PDW and MPV showed significant differences in all three evaluation periods. A direct relation between PDW and MPV is described in healthy humans. This relation is kept in patients with thrombocytopenia derived from peripheric destruction, such as the one observed in horses of the present study due to the partake in bleeding procedures. It is then explained why both parameters were kept high and within the reference ranges for the equine species. If thrombocytopenia was derived from a production deficit, however, it would be observed an increase in PDW and a decrease in MPV, since PDW depends on the platelet production by megakaryocyte fragmentation (Buttarello and Plebani, 2008). A decrease in PLT, therefore, could explain the dramatic decrease in P-LCR observed in T2.

Blood biochemical panel is a resource for defining the metabolic status of animals, providing information regarding organ malfunction, tissue injury, adaptation in face of nutritional or physiological challenges, and during some specific metabolic dysfunctions. Therefore, blood biochemistry can provide grounds for the interpretation of liver, kidney, pancreas, bone, and muscular physiological status. TP, AST, GGT, and AL can provide relevant information regarding liver function, whereas urea and creatinine can be associated with kidney lesions (Gonzáles et al., 2006). Thus, biochemical findings are described in Table 4.

Table 4. Blood biochemical panel of horses that underwent immunization with *Loxosceles* spp. venom on three different evaluation periods (mean ± SEM).

Analyte	Evaluation period			Reference values for horses[1]
	T0	T1	T2	
Urea (mg/dL)	35.8±1.33	36.3±5.40	32.6±1.16	21.4-51.5
Creatinine (mg/dL)	1.36±0.11 a	1.32±0.09 a,b	1.09±0.05 b	0.4-2.2
ALT (U/L)	4.32±1.07	4.72±0.87	5.29±0.77	3.0-23.0
AST (U/L)	155.3±10.6 a	95.1±10.9 b	94.5±8.71 b	226-336
ALP (U/L)	159.3±12.9	144.0±17.2	164.4±12.4	86.0-295.0
GGT (U/L)	10.4±1.32 a,b	17.4±4.22 a	5.94±0.68 b	6.0-32.0
Glucose (mg/dL)	101.3±7.97 a	72.8±4.19 b	103.5±3.65 a	62.0-134.0
Amylase (U/L)	7.79±2.92 a	1.65±0.54 b	2.32±0.66 b	14.0-35.0
TP (g/dL)	8.90±0.46	9.24±0.83	8.91±0.24	6.0-8.0
Albumin (g/dL)	3.63±0.23 a	2.51±0.11 b	2.31±0.07 b	2.4-4.1
Globulins (g/dL)	5.28±0.35	6.73±0.77	6.60±0.27	2.6-4.0
Cholesterol (mg/dL)	91.4±5.89 a	59.2±2.18 b	59.1±1.92 b	75.0-150.0
Triglycerides (mg/dL)	42.9±3.02 b	48.3±2.64 b	67.9±3.80 a	4.0-44.0
Lactate (mg/dL)	18.4±1.78	37.7±5.53	55.0±29.3	10.0-16.0
LDH (U/L)	306.2±40.3	253.5±26.1	278.0±25.6	162.0-412.0

Alanine aminotransferase (ALT); aspartate transaminase (AST); alkaline phosphatase (ALP); gamma-glutamyl transpeptidase (GGT); total protein (TP); and lactate dehydrogenase (LDH).
[1]Reference values according to Laboratory of Clinical Analyses, Veterinary Hospital, UFMG.
a,b,c: Different letters between evaluation periods show significant difference ($p<0.05$, mixed linear model)

Creatinine plasmatic levels and urea were not altered during the evaluation periods. Creatinine is a good indicator of glomerular filtration rate since it is not altered by tubular activity (Toribio, 2007). These results are very positive and paramount in order to assure renal function and, henceforth, animals' longevity in the antivenom procurement scale. A primary concern regarding horses as antivenom producers is its immune complexes synthesis. Since the main goal of these animals is to produce high levels of IgG, they receive high amounts of antigen, and consequently might produce large amounts of soluble immune complexes. These immune complexes can circulate and deposit in the glomeruli, as well as the synovium of joints and capillaries, thus causing glomerulonephritis, arthritis, and vasculitis, respectively (DeFranco et al., 2007).

Despite only albumin presented statistical significance during the evaluation periods, it is valid to comment TP and globulin results. Globulins sustained a pattern of increase during all evaluation periods, and albumin a decrease. These results were already expected since the inoculated antigens and adjuvants employed in the immunization protocols are responsible for inducing an immunologic response which, in turn, produces

immunoglobulins (DeFranco et al., 2007) and reflects on an increase in globulin fractions. TP might not have had statistical significance due to a decrease in albumin, but animals presented hyperproteinemia. Hyperproteinemia can be attributed to increase in globulin levels as well as immunocomplex formation due to antigen/antibody reaction (Gade and Khadilkar, 2005). In an acute phase reaction condition, such as the one horses of the present study have undergone, gamma-globulins, serum amyloid A, alpha-macroglobulin and hepatoglobulins can be increased, as previously reported by Husby (1992).

Amylase suffered a significant decrease ($p<0.05$) between T0 and the subsequent evaluation periods. Amylase activity is directly connected to pancreatic function, and low levels of amylase might be correlated to a decrease in starch digestion by the small intestine (Richards et al., 2016). This impairment in starch digestion might explain the significantly higher levels of triglycerides ($p<0.05$) found in T2. Low serum amylase activity is also correlated with metabolic syndrome (Kei et al., 2011). Horses of the present study kept elevated body scores (4/5 and 5/5) during all sampling moments and had characteristic adiposity of Equine Metabolic Syndrome, such as neck crest and tail adiposity (Mccue et al., 2015). The higher triglycerides mean values of these horses regarding reference ranges for the species also corroborate with this hypothesis.

Cholesterol showed a significant decrease ($p<0.05$) between T0 and the remaining evaluation periods. These results are in accordance with those found by Betiol et al. (2008) while evaluating horses under diphtheria antivenom production. These authors found a similar cholesterol decrease after periods of immunization and bleeding. Liver is the main responsible for cholesterol synthesis (Guyton and Hall, 2015) and can present disfunction when the body suffers a severe inflammatory process, such as the one horses of the present study have undergone. Protein synthesis is also a liver function, and hyperproteinemia has already been previously discussed in this section. Other enzymes that can mirror liver function are ALT, AST, GGT, and even ALP. Impairment or dysfunction of this organ is generally illustrated by severe increases in these enzymes, which was not observed in the present study. Similarly to what was already commented in this section regarding renal function, it is paramount that horses maintain a healthy liver in order to assure their longevity in the antivenom producing process, and the results mentioned above are a positive indicator of horses' health and its upkeep during all immunization cycles.

3.3 Electrocardiography results

Loxosceles spp. venom can exert a direct cardiotoxic effect (Dias-Lopes et al., 2010). Hence, ECG recordings were made since arrhythmias can be diagnosed by clinical examination but might require complementary exams in order to reach a reliable diagnosis (Durando, 2003; McSloy, 2011; Menzies-Gow, 2015). Apart from thrombophlebitis, no significant cardiovascular alterations were diagnosed in T0 and T1; however, on T2 eight horses (72.73%) presented some sort of unconformity during cardiac auscultation, especially splitting of the first (S1) or second (S2) heart sound. S1 occurs at the beginning of ventricular systole and represents the closure of atrioventricular valves. S2 splitting is an asyncronous closure of semilunar valves and is considered physiological in horses (Reef et al., 2014). Variation in the intensity and quality of S1 are uncommon in horses (Schwartzwald, 2013), but a bleeding of such magnitude (21 L for antivenom production) might justify this finding. S1 split can come from a prolonged diastolic period and arrhythmias (such as the ones diagnosed in the present study) (Speirs, 1992). Thus, ECG results are presented in Table 5.

Table 5. Electrocardiography parameters of horses that underwent immunization with *Loxosceles* spp. venom on three different evaluation periods (mean ± SEM).

ECG parameter	Evaluation periods			Reference values for horses[1]
	T0	T1	T2	
Heart rate (bpm)	56.0±3.80 a,b	45.7±1.29 b	58.9±3.75 a	28-40
P (ms)	118.8±6.50	126.5±4.41	115.5±5.85	<160
P (mV)	0.43±0.02 a	0.34±0.03 b	0.42±0.02 a	
PR (ms)	282.8±20.6	293.7±10.1	264.8±17.3	<500
QRS (ms)	128.3±6.93	139.0±4.20	129.3±3.95	<140
R (mV)	1.03±0.15 b	1.76±0.17 a	1.63±0.17 a	
QT (ms)	434.2±18.8 b	483.1±10.2 a	436.5±14.1 b	<600
T (mV)	0.46±0.04 b	0.79±0.05 a	0.76±0.08 a	

[1]Reference values according to Menzies-Gow (2015)
a,b,c: Different letters between evaluation periods show significant difference ($p<0.05$, mixed linear model)

As previously explained in section 3.2, severe bleeding (as the one performed before T2 sampling) can reduce arterial and capillary pressure, inciting a sympathetic response. Thus, baroreceptors and chemoreceptors will induce the secretion of catecholamines by the adrenal medulla, promoting an increase in heart rate and contractility (Guyton and Hall, 2015). This effect might explain why heart rate in T2 showed higher significant values compared to T1.

At ECG examination of present study, horses presented normal sinus as the predominant rhythm (Fig. 4.), as well as sinus arrhythmia, and sinus tachycardia (Fig. 5).

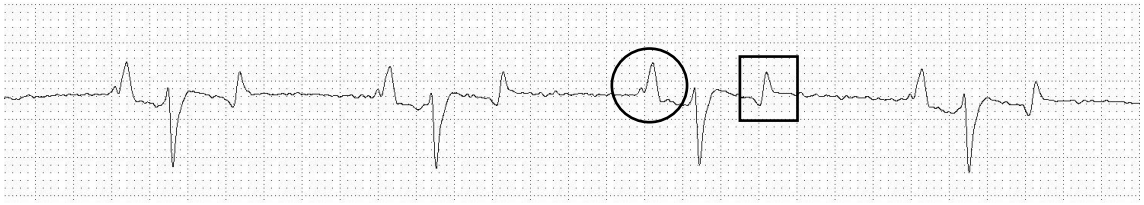

Figure 4. Normal sinus rhythm ECG from a horse housed at Centro de Produção e Pesquisa de Imunobiológcos (Brazil) in T0 (immunization yet to be performed). Common findings in the majority of ECG recordings were bifid P (circle) wave and biphasic T wave (square).

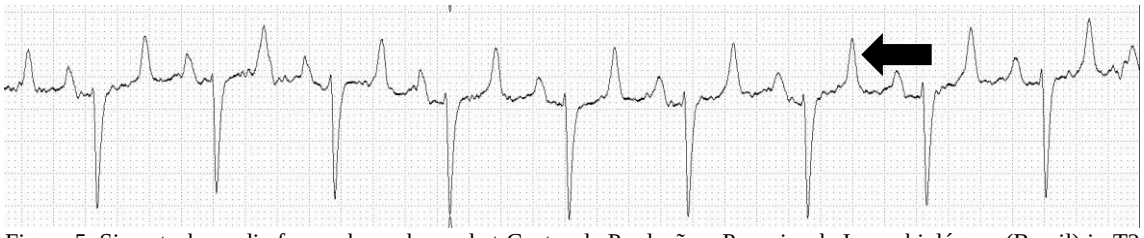

Figure 5. Sinus tachycardia from a horse housed at Centro de Produção e Pesquisa de Imunobiológcos (Brazil) in T2 (after commercial bleeding was performed). An increase in T wave is also observed (arrow).

Arrhythmias were diagnosed in two horses (18.18%) only on T2, consisting of an advanced second-degree atrioventricular block (AV) (Fig. 6) in which normal QRS complexes are interspersed by periods where P waves are not followed by QRS complexes, though maintaining P-P intervals. Periods of atrioventricular block can be prolonged (Menzies-Gow, 2015). Clinical relevance of such findings, however, is difficult to be determined. Clinically healthy horses at rest can manifest second-degree AV-block (Raekallio et al., 1992), credited to fluctuations in autonomic tone (Guyton and Hall, 2015) as horses naturally exhibit a high vagal tone (Speirs, 1992).

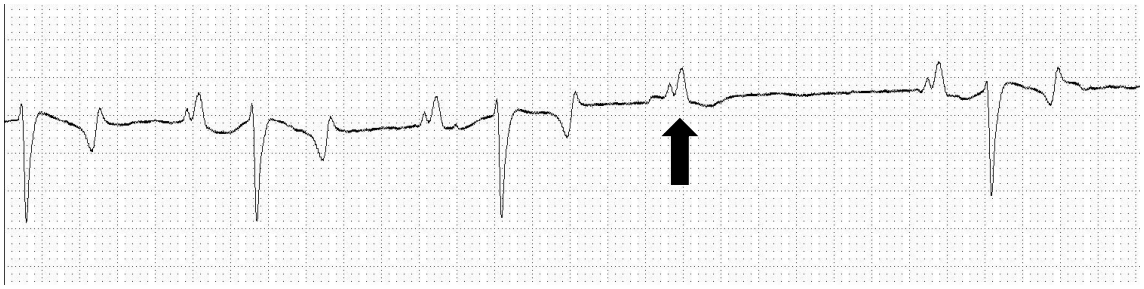

Figure 6. Second-degree atrioventricular block from a horse housed at Centro de Produção e Pesquisa de Imunobiológcos (Brazil) in T2 (after commercial bleeding was performed).. Observe a P wave (arrow) with no following QRS complex.

Intervals of PR, QRS, and QT showed no statistical difference between evaluation periods and were kept within the reference values for the species in all periods. P, T, and R waves were statistically different ($p<0.05$) between T0 and the remaining evaluation periods, with a tendency of mean increase from T0 to T2. T wave represents ventricle repolarization, and its alterations can often be related to electrolyte imbalance (such as hyperkalemia) as well as coronary vasospasm (Hesselkilde et al., 2014). An increase of alpha-adrenergic receptor activity in epicardial coronary arteries or excessive catecholamine release that may activate these receptors can lead to coronary vasospasm (Verouden et al., 2009). An increase in R waves might be connected to myocardial compromise, such as ischemia, as well as tachycardia, since both alter electric heart conduction. Horses had no statistical difference in heart rate but kept bpm over the reference values for the species, thus presenting tachycardia. Another finding that corroborates with myocardial ischemia is a biphasic T wave (Guyton and Hall, 2015). Due to loxoscelic venom cardiotoxicity (Dias-Lopes et al., 2010), it could be inferred that some level of ischemia might have led to these alterations. Ischemia is also originated from bleeding for antivenom procurement, since hypovolemia occurred after this procedure. Nonetheless, several biphasic T waves were also recorded in T0, where horses had yet not gotten in touch with loxoscelic venom or suffered bleeding.

Another interesting finding in all three evaluation periods was P wave morphology. The P wave represents atrial depolarization, and the majority of horses presented a bifid P wave (Fig. 4) from T0 to T2. Horses are large animals, with an enormous atrial muscular mass that needs to be depolarized for the cardiac cycle to start. Thus, it is a particularity of the species to physiologically have a bifid P wave on the ECG, since depolarization might suffer a small delay due to the large mass that needs to undergo depolarization (Durando, 2003; Menzies-Gow, 2015).

Determining the clinical impact of all the ECG findings mentioned above is a difficult task. Since most of the alterations were diagnosed in T2, it is possible that hypovolemia caused by bleeding for antivenom procurement could exert some pathological effect on horses' physiology.

4 Conclusions

Loxoscelic immunization protocol has impacted the horses' health, especially after bleeding for commercial antivenom procurement. Despite a significant decrease in RBC, PCV, and hemoglobin in T2, horses kept liver and renal functions within physiological levels. ECG was able to record some arrhythmias, but their real clinical impact remains unclear. Thrombophlebitis and subcutaneous abscesses were the main clinical findings, but are inherent to antivenom procurement management, due to the systematic venipunctures and use of adjuvants at the venom inoculation sites, respectively.

CHAPTER 4

Long-term effects of *Loxosceles* spp. immunization protocols in horses

Abstract

Antivenom production relays on horses being immunized and bled for plasma harvest. One horse can partake in several complete cycles of antivenom production, which will take years of constant venom and adjuvant inoculation and bleedings. It is unknown the true impact on horses' health that partake in several antivenom producing cycles. Therefore, the aim of this study is to evaluate horses that have undergone several complete cycles of loxoscelic antivenom production. Seven crossbred horses, who had partaken in six or more complete antivenom-producing cycles were used and established as the immunized group (IG). Eleven horses, under same handling and general management, were established as the control group (CG). Horses were evaluated regarding their general clinical status, had their blood sampled and an electrocardiographic examination (ECG) recorded. IG presented lower red blood cell count and packed cell volume, despite keeping values within inferior limits for the species. Renal function was not impaired but liver-related enzymes were higher when compared to CG, probably due to liver exertion from immunoglobulin synthesis. ECG showed some abnormalities in IG, corroborated by increase in creatine kinase/isoenzyme MB fraction (CK-MB). Cardiovascular abnormalities were mainly found in horses partaking in several antivenom producing cycles, but animals kept an acceptable general health status.

1. Introduction

Antivenom remains the most suitable treatment for loxoscelism, a syndrome triggered by spider bite from *Loxosceles* spp. (Pauli et al., 2006; WHO, 2006). The most common clinical manifestation of loxoscelism is cutaneous, consisting of a progressive dermonecrotic lesion with gravitational spreading. In a lesser extent, loxoscelism can manifest itself in its viscero-cutaneous form, consisting of systemic hemolysis, intravascular coagulation and acute renal failure, often leading to death (Swanson and Vetter, 2006; Silva et al., 2009; Chaim et al., 2011). Antivenom production is based on immunizing animals, harvesting their blood, purifying it and procuring an immunobiological filled with immunoglobulins to counteract and neutralize envenomation deleterious effects (Hogan et al., 2004; De Roodt et al., 2007; Gutiérrez et al., 2007; Gutiérrez et al., 2009; Waghmare et al., 2014; Calabria et al., 2019).

Despite its obvious public health importance, antivenom procurement faces several hindrances, ranging from venom extraction to animal welfare (Angulo et al., 1997; Gutiérrez et al., 2007; Gutiérrez et al., 2009). The latter is especially relevant for horses, since they are the main species used for antivenom procurement (Pratanaphom et al., 1997; Malikides et al., 2001; Hogan et al., 2004; Waghmare et al., 2014), thus, antivenom production plays an important role in public health, especially in Brazil, where antivenom in loxoscelism is largely employed (Pauli, 2008; Ministério da Saúde, 2019).

Therefore, the aim of this study is to evaluate aspects regarding the general health of horses used in several immunization cycles for loxoscelic antivenom procurement, comparing clinical, hematological and electrocardiographic findings with those of naïve horses.

2. Material and Methods

2.1 Ethical statement

All procedures involving horses were conducted according to animal welfare guidelines after approval by the Ethical Committee for the Use of Animals of the Federal University of Minas Gerais (CEUA/UFMG), under protocol number 159/2019 (Annex I).

2.2 Venom

Venom was obtained from spiders captured within Paraná and Santa Catarina states, Brazil, and kept under controlled conditions in Center of Production and Research of Immunobiologicals (CPPI), Piraquara, Paraná state. Specimens of *L. intermedia*, *L. gaucho* and *L. laeta* had their venom extracted after being restrained from feeding for 30 days and undergoing an electrical stimulus of 12 V applied on the cephalothorax region. The venom pool obtained was dehydrated and kept on -20°C, in the dark, until its use. An electrophoretic pattern of *L. intermedia* venom was described previously in Chapter 3 (Fig. 3).

2.3 Horses and experimental groups

Seven healthy crossbred horses, both male and female, weighting 501.86±95.29 Kg and of 18.86±3.34 years-old were used. Horses belonged to and were housed on the CPPI. Animals were kept in pasture, and had access to 6 Kg of alfalfa hay, 2 Kg of a 12% protein commercial equine ration, plus 2 Kg of hydrated/germinated oat grains per day. Mineral salt and fresh water were also provided *ad libitum*. Horses were acquired from certified properties free of glanders and Infectious Equine Anemia. Horses were vaccinated against tetanic toxoid, leptospirosis, strangles, influenza, rabies, and encephalomyelitis, and then dewormed.

Horses here evaluated have already undergone six or more complete immunization cycles for loxoscelic antivenom production and named immunized group (IG). This horses' first immunization protocol followed the same described for naïve horses in Chapter 3 (Table 1), and the consecutive ones consisted only of reimmunization cycles, since hyperimmunization is performed only once during the horses' lives. When horses acquired a satisfactory immunoglobulin titrate, bleeding for antivenom industrial procurement was then performed. When horses were evaluated in this experiment, they have not undergone any sort of bleeding or immunization for at least 60 days and some were already being retired from their activities.

For the control group (CG), 11 naïve horses were used. These horses were not used for any sort of immunization protocol but were kept under the same nutritional and general management than the experimental group, as well as purchased from the same suppliers and handled daily in a similar manner.

2.4 Clinical and hematological evaluation

Horses were restrained using a halter and examined inside a stock. No sedation was needed. Horses underwent a thorough physical examination, encompassing inspection, measurement of parametric indexes and clinical evaluation of biological systems, according to Speirs (1992). A form was previously designed and fulfilled accordingly (Annex II)

Blood sampling was performed after antisepsis of the external jugular vein region using 70% alcohol. Tubes containing ethylenediaminetetraacetic acid (EDTA) and clot activator were used (*BD Vacutainer®-Becton Dickinson*) in order to perform hematologic

and biochemical analyses, respectively. Hematimetric indexes were obtained using an automatic cell counter (*pocH-100Iv-Diff-Sysmex®*) with a software calibrated for the equine species; serum biochemistry was analyzed by an automatized biochemistry equipment (*Cobas Mira Plus®*) under a clinical pathologist supervision. Blood parameters evaluated were as follows: red blood cell count (RBC); packed cell volume (PCV); white blood cell count (WBC); red blood cell distribution width (RDW); lymphocytes, and sum of other WBC, such as neutrophils, monocytes and basophils (OTH); total platelet count (PLT); mean platelet volume (MPV); platelet distribution width (PDW); platelet clump (P-LCR); urea; creatinine; alanine aminotransferase (ALT); aspartate transaminase (AST); alkaline phosphatase (ALP); gamma-glutamyl transpeptidase (GGT); glucose; amylase; total proteins (TP); albumin; globulins; cholesterol; triglyceride; lactate; lactate dehydrogenase (LDH); creatine kinase (CK); and creatine kinase/isoenzyme MB fraction (CK-MB).

2.5 Electrocardiographic evaluation

Horses underwent an ECG evaluation using a portable 12-channel digital electrocardiograph (*TEB ECG Vet® - Tecnologia Eletrônica Brasileira S.A.*). ECG recordings were acquired in a quiet environment and with horses in orthostatic position. Electrodes were fixed using alligator metal clips embedded in alcohol and attached to the horses' skin using Dubois configuration (Costa et al., 2017), in which electrodes 1 and 2 are placed next to the spine tuberosity of both left and right scapula, electrode 3 on the xiphoid process of the sternum and electrode 4 on the proximal cranial region of the left forelimb. Recordings were made at 25 mm/sec speed and sensitivity of 1 cm = 1mV. Bipolar (DI, DII, DIII) and augmented unipolar (aVR, aVL, aVF) leads were recorded. The following parameters were evaluated: cardiac rhythm; cardiac frequency; P (ms); P (mV); PR, QRS and QT intervals; R and T waves and ST segment levels. The electric axis was determined by QRS polarity in the bipolar and unipolar augmented leads.

2.6 Statistical analysis

The statistical analyses were performed using R software (version 3.6.1). The normality of the data was evaluated using the Shapiro-Wilk test. Student t-test was employed for the comparison of parametric data, and Mann-Whitney test for comparison of non-parametric data. The level of statistical significance was set at $P < 0.05$.

3. Results and discussion

3.1 Clinical features

All horses presented a satisfactory clinical condition, since no major clinical alterations were diagnosed. Some horses from the CG needed a twitch to ensure the handler's safety. This restraining method might explain why horses from the CG, despite no statistical significance, presented higher heart rates (56.0±3.80 bpm) than those of the IG (45.9±5.81 bpm). Major clinical differences between groups were the presence of multiple subcutaneous abscesses and bilateral thrombophlebitis in IG. None of these alterations was seen in CG, since no horses had gotten in touch with adjuvants, venom, inoculations, or bleeding procedures. Subcutaneous abscesses have already been discussed in Chapter 3 and occur mainly due to the adjuvants use and the inflammatory response caused by the loxoscelic immunization protocol (Apostólico et al., 2016). Similarly, thrombophlebitis occurred due to the systematic venipunctures on the external jugular vein that horses from the IG have undergone (Barakzai and Chandler, 2003; Divers, 2003; Milne and Bradbury, 2009).

All horses from IG presented lymphadenopathy, whereas none of the CG presented any sort of lymph node enlargement. In the present study, lymph nodes such as mandibular, parotid, retropharyngeal, superficial cervical and ischiatic were palpable, with increased temperature and slight sensibility. These characteristics are notable in horses under a general inflammatory process (Speirs, 1992). Lymph nodes are clusters of germinal cells within a connective tissue framework, containing lymphocytes and macrophages in close proximity, playing a key role in the immune response. Inflammation, such as the one incited by the adjuvants and venom used in the present study, can cause lymphadenopathy due to lymphocyte proliferation and accumulation of antigenic materials (DeFranco et al. 2007).

3.2 Hematologic and biochemical analyses

Blood cells can reflect several physiological and pathological variations happening in organs or the body systems in general. More often, it can portray a general response of the individual to both pathological and physiological conditions. Interpretation of hematological profiles should always occur concurrent to the horse's history and physical findings, thus directing the clinician to results true significance (Satué et al., 2012). Results from CBC are shown in Table 6.

Table 6. Complete blood count panel of immunized horses using *Loxosceles* spp. venom and of naïve horses (control group) (mean ± SEM).

Parameter	Control group	Immunized horses	Reference values for horses
WBC (cell x 10^3/µL)	12.0±0.67	9.66±0.83	5.20-13.9[1]
RBC (cell x 10^6/µL)	8.56±0.32a	6.61±0.28 b	6.4-10.0[1]
PCV (%)	39.5±1.67a	33.9±1.31 b	32.0-47.0[2]
MCV (fL)	46.0±0.44b	51.4±1.16 a	37.0-59.0
PLT (cell x 10^3/µL)	127.9±13.0a	113.4±10.5 b	120.0-256.0[1]
Lymphocytes (%)	35.1±2.15a	30.1±1.90 b	
OTHR (%)	64.9±2.15b	69.9±1.90 a	
Eosinophils (%)	0.00±0.00	0.43±0.43	
Lymphocytes (cell x 10^3/µL)	4.14±0.29a	3.03±0.23b	1.5-7.7[1]
OTHR (cell x 10^3/µL)	9.62±1.99	6.83±0.75	
Eosinophils (cell x 10^3/µL)	0.00±0.00	0.04±0.04	
RDW-SD (fL)	37.7±0.55b	44.6±1.12 a	
RDW-CV (%)	20.7±0.25b	22.3±0.40 a	21.0-25.0[1]
PDW (fL)	10.2±0.32	12.0±1.10	
MPV (fL)	8.29±0.13	9.07±0.38	5.3-7.8[2]
P-LCR (%)	7.68±1.00	17.9±4.46	

Red blood cell count (RBC); packed cell volume (PCV); mean corpuscular volume (MCV); white blood cell count (WBC); red blood cell distribution width (RDW); sum of other WBC, such as neutrophils, monocytes and basophils (OTHR); total platelet count (PLT); platelet distribution width (PDW); mean platelet volume (MPV); platelet clump (P-LCR).

[1]Reference values according to Grondin and Dewitt (2010)

[2]Reference values according to Laboratory of Clinical Analysis, Veterinary Hospital, Cardenal Herrera University

a,b: Different letters between groups show significant difference ($p<0.05$, Student t test)

Despite remaining within the reference values of the species, IG horses kept variables such as RBC and PCV on inferior limits. Age can be a relevant factor that can influence these parameters and horses from IG here evaluated were 15 years-old or older, whereas those of CG ranged from 5 to 8 years-old. McFarlane et al. (1998) and Satué et al. (2009) found a reduction of RBC, with compensatory increase of MCV, in older horses, probably due to an impaired regenerative capacity of the bone marrow of geriatric horses. In the

present study, both MCV was superior in the IG when compared to the CG, which corroborate with previous findings (Satué et al., 2009). Thus, it can be inferred that horses might present some level of anemia, perhaps due to the partake in numerous and systematic bleedings for antivenom procurement, as well as an impaired regenerative bone marrow capacity. However, RDW, a coefficient of the anisocytosis degree of RBCs, was not increased (22.30 ± 0.40%), which attest IG horses CBC normality regarding its erythron parameters. This RDW index can also lead to another inference. Perhaps, this PCV and RBC difference between experimental groups were narrower. PCV can be unstable in the horse, due to its unique spleen capacity of retaining, at rest, from 6 to 12 L of erythrocytes-rich blood (McKeever et al., 1993). In response to excitement, such as handling, twitching, any sort of pain and even venipuncture itself, a catecholamine release can induce RBCs' transfer from spleen to circulation (Kunugiyama et al., 1997). In the present study, the handling of horses was carefully conducted. However, as previously mentioned, horses from CG were younger and more stressed, thus needing more energic physical restraint methods, which might have caused catecholamine release and spleen contraction, perhaps overvaluing PCV and RBC indexes.

PLT were significantly different between CG and IG, but no animal was diagnosed with thrombocytopenia, since most laboratories define thrombocytopenia as a PLT below 100.000/µl. Because accumulation of platelets is common in horses, the blood smear should always be evaluated to confirm the actual decrease in platelets and to prevent the existence of a pseudothrombocytopenia, which was also done in the present study. P-LCR, an index which infers presence of platelet clumps, and MPV showed no statistical significance between groups, thus corroborating with PLT statistical significance. The decrease in the number of circulating platelets may be the result of decreased bone marrow production, increased destruction, or increased platelet use during coagulation (Brooks, 2008; Satué et al., 2017). According to Brooks (2008), several pathologies can outcome in increased consumption or loss of platelets in horses, such as vasculitis, renal and gastrointestinal diseases. Horses from IG of the present study presented thrombophlebitis, which might influence of platelet consumption and contribute to the statistical difference seen regarding this index. Furthermore, no renal diseases were present, since no difference of urea or creatinine were established between experimental groups and values were kept within reference ranges for the species (further discussed on section 3.3 of this chapter). Horses from the IG group, however, have systematically

suffered several blood-withdraws during their lives. According to Dodds (1997), the thrombocytopenia effects of such procedures are rapidly reversible, corroborating with findings described by Malikides et al. (2001). Since IG horses have not undergone any procedure for at least 60 days, the maintenance of PLT within reference ranges is justified.

Despite no statistical difference between WBC was found, a significant difference ($p<0.05$) was seen between lymphocytes and neutrophils in the experimental groups. A slight neutrophilia pattern was observed on the IG, which disrupted the 2:1 neutrophil:lymphocyte ratio commonly observed in the equine species (Dodds, 1997). This 2:1 pattern was maintained in the CG. This increase in neutrophils in horses from the IG might be due to the several immunization-protocols these horses have undergone, with effects of adjuvants and even of venom toxicity itself. However, since neutrophils are largely recruited in acute inflammation (Dodds, 1997), maintenance of several inflammatory focuses, such as thrombophlebitis and subcutaneous abscesses, might be enough to justify this discrete neutrophilia and its contribution to disrupting neutrophil:lymphocyte proportion.

Main blood biochemical parameters obtained from experimental groups are shown on Table 7.

Table 7. Blood biochemical parameters of immunized horses using *Loxosceles* spp. venom and naïve horses (control group) (mean ± SEM).

Parameter	Control group	Immunized horses	Reference values for horses[1]
Urea (mg/dL)	35.8±1.33	36.3±3.58	21.4-51.5
Creatinine (mg/dL)	1.36±0.11	1.16±0.06	0.4-2.2
ALT (U/L)	4.32±1.07b	16.4±2.68a	3.0-23.0
AST (U/L)	155.3±10.6b	223.7±10.9a	226-336
ALP (U/L)	159.3±12.9b	237.5±29.6 a	86.0-295.0
GGT (U/L)	10.4±1.32d	24.0±9.22 c	6.0-32.0
Glucose (mg/dL)	101.3±7.97	115.7±11.7	62.0-134.0
TP (g/dL)	8.90±0.46	7.81±0.20	6.0-8.0
Albumin (g/dL)	3.63±0.23c	2.18±0.08d	2.4-4.1
Globulin (g/dL)	5.28±0.35	5.63±0.26	2.6-4.0
Cholesterol (mg/dl)	91.4±5.89	79.3±2.96	75.0-150.0
Triglicerydes (mg/dl)	42.9±3.02a	19.9±2.86 b	4.0-44.0

Alanine aminotransferase (ALT); aspartate transaminase (AST); alkaline phosphatase (ALP); gamma-glutamyl transpeptidase (GGT); total protein (TP).
[1]Reference values according to Laboratory of Clinical Analyses, Veterinary Hospital, UFMG.
a,b: Different letters between groups show significant difference ($p<0.05$, Student t test)
c,d: Different letters between groups show significant difference ($p<0.05$, Mann-Whitney test)

The serum activities of all enzymes related to liver function, such as ALT, AST, GGT, and ALP, showed statistical significance between experimental groups. IG presented higher values for these variables but kept them within reference ranges for the species. According to Betiol et al. (2008), horses that are antivenom producers have a higher hepatic demand, since liver is responsible for synthetizing globulins. However, in order to indicate a dysfunction or impairment, severe increases in these enzymes should be observed (Dodds, 1997), which was not the case. Similar results were obtained from Waghmare et al. (2014), while evaluating horses used for snake antivenom production. These same authors have also found no kidney impairment, as in the present study, in which urea and creatinine were kept within reference ranges and had no statistical significance between IG and CG.

Horses had a significant difference between albumin levels, in which IG presented lower values than CG. Decrease in albumin levels (but maintenance within physiological ranges) in horses that are antivenom producers have been previously described (Betiol et al., 2008; Waghmare et al., 2014). This decrease might be attributed to inflammation, a reaction horses of IG have systematic been exposed to. Inflammation stimulates the production of cytokines, especially IL1, IL6 and TNF-α, that act on the liver, in order to

produce new acute phase proteins, such as serum amyloid A and fibrinogen. Consequently, albumin production is impaired (Netto et al., 2004).

Triglycerides levels were also significant different ($p<0.05$) between experimental groups. CG presented higher values, and some animals of this group presented triglycerides levels above reference values for the species. Horses from the CG presented higher body scores and weight than those of IG, and had visible adiposity deposits, such as neck crest, which might explain its higher triglycerides levels and the higher cholesterol tendency (McCue et al., 2015).

3.3 Electrocardiographic alterations

There is a cardiotoxicity related to *Loxosceles* spp. venom (Dias-Lopes et al., 2010) and ECG recordings can be helpful in detecting and grading some arrhythmias and myocardial injuries, often overlooked during clinical examination (Speirs, 1992; McSloy, 2011; Menzies-Gow, 2015). According to Robertson (1992) and Menzies-Gow (2015), horses present a high incidence of cardiac arrhythmias and several of them holds a physiological background, such as first and second-degree AV block, pacemaker migration and sinus bradycardia. However, premature atrial or ventricular complexes, advanced secondo or third-degree AV blocks and atrial fibrillation can directly affect performance.

All horses in CG presented normal sinus rhythm. From the seven horses of IG, six presented some conduction abnormality, such as first-degree AV block (Fig. 7) (42.86%), polymorphic ventricular tachycardia (14.29%) (Fig. 8), pacemaker migration (14.29%) (Fig. 9), second-degree AV block (14.29%) (Fig. 10), sinus tachycardia (14.29%) and sinus arrhythmia (14.29%).

Sinus arrhythmia can normally be diagnosed in horses at rest (as well as sinus bradycardia, not found on the present study), due to horses' high vagal tone. Sinus tachycardia, if not accompanied by other clinical findings, might be related to excitement due to handling or stress (Speirs, 1992; Menzies-Gow et al., 2015; Bomfim et al., 2017).

First-degree AV block is a common finding in horses' ECG, due to the higher vagal tone of the species, and can disappear after exercise (Speirs et al., 1992). However, hyperkalemia or drug administration might also cause it (Schwartzwald, 2013). This conduction abnormality is diagnosed when PR interval exceeds 0.2 seconds, which means

that conduction from sinus node to ventricles is prolonged (Menzies-Gow, 2015). Clinical significance, however, is noted only when PR intervals are extremely prolonged, over 0.5 seconds (Raekallio, 1992; Hasselkilde et al., 2014). First-degree AV block was the main finding in the ECGs of the present study, since it encompassed over 42% of the animals evaluated. Despite PR increase (but all under 0.5 seconds), no clinical significance of this finding was highlighted, corroborating with its possible physiologic origin.

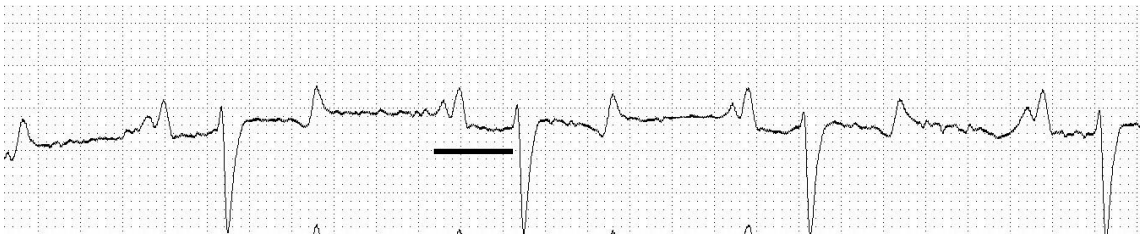

Fig. 7 First-degree AV block on a horse who has undergone several immunizations for loxoscelic antivenom production. Note increase PR interval (0.35s) (black line).

Polymorphic ventricular tachycardia is defined by four or more consecutive ventricular beatings, and can be an indicative of primary myocardial disease, hypoxia or electrolyte imbalance (Schwartzwald, 2013), and was described in rattlesnake envenomation. Clinical signs of congestive heart failure should be observed, since they become more severe during time, due to shorter cycle lengths, higher heart rates and polymorphic ventricular rhythm. Polymorphic ventricular tachycardia is associated with increased electrical inhomogeneity and instability, thus increasing the risk of a fatal rhythm to develop.

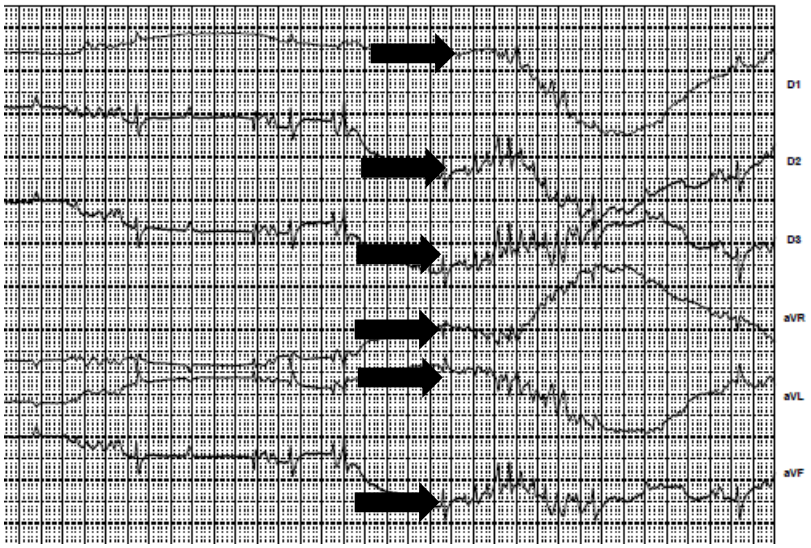

Fig. 8. Polymorphic ventricular tachycardia observed in one horse who have partaken in several *Loxosceles* spp. immunization protocols for antivenom production. Observe that over four consecutive ventricular beatings occurred in all bipolar and augmented unipolar leads (arrows).

Pacemaker migration occurs when different P wave amplitudes are seen and was also observed in one horse. This conduction abnormality can be caused by a higher vagal tone and is considered a physiological finding, previously described by several authors (Durando, 2003; Diniz et al., 2011; McSloy, 2011; Bello et al., 2012; Bomfim et al., 2017).

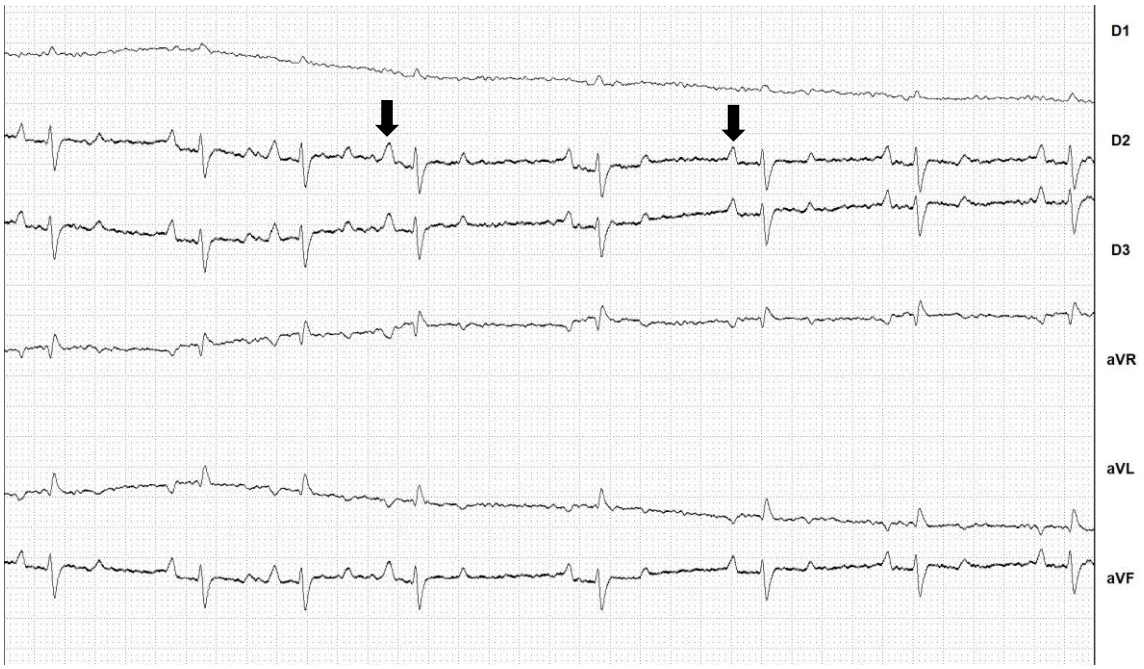

Fig. 9. Sinus arrhythmia accompanied by peacemaker migration observed in one horse who have partaken in several *Loxosceles* spp. immunization protocols for antivenom production. It occurred in all bipolar and augmented unipolar leads. Observe different P wave amplitudes (arrows)

Second-degree AV block was also seen in one horse. As previously discussed, this finding is common in clinically healthy horses at rest (Raekallio et al., 1992; Diniz et al., 2011; Bomfim et al., 2017), credited to fluctuations in autonomic tone (Guyton and Hall, 2015) as horses naturally exhibit a high vagal tone (Speirs, 1992).

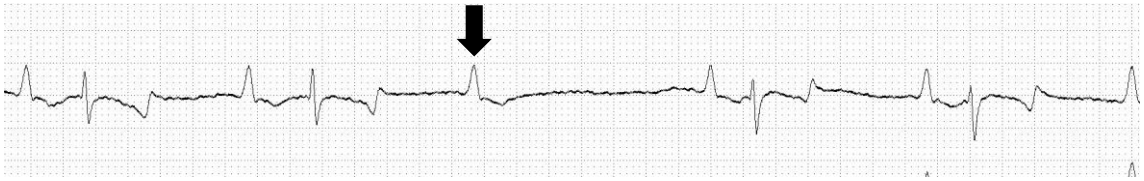

Fig. 10 Second-degree AV block. Note that a P wave is not followed by a QRS complex (arrow), despite maintenance of PP intervals.

Results from ECG recordings are presented on Table 8. Only QT intervals and T wave showed statistical differences. QT interval represents cardiac repolarization, and an increase was observed in IG regarding CG. QT might be influenced at some extent by heart rate, body weight, sex, autonomic tone, and environment. Horses might as well present a considerable inter-breed variation for this variable (Pedersen et al., 2016), which might explain the difference observed in the present study. All horses were crossbreds and CG was composed of younger horses. Despite this statistical difference, it is important to highlight that both experimental groups kept QT values within the reference range for the species and no relevant clinical finding was correlated with this parameter variation.

Table 8. ECG results from horses used in several immunization protocols from loxoscelic antivenom production and naïve horses (control group) (mean ± SEM).

ECG parameter	Control group	Immunized horses	Reference values for horses[1]
HR (bpm)	56.0±3.80	45.9±5.81	28-40
P (ms)	118.8±6.50	133.6±10.9	<160
P (mV)	0.43±0.02	0.43±0.04	
PR (ms)	282.8±20.6	297.0±18.2	<500
QRS (ms)	128.3±6.93	133.7±5.03	<140
R (mV)	1.03±0.15	0.73±0.11	
QT (ms)	434.2±18.8b	509.3±23.2 a	<600
T (mV)	0.46±0.04 a	0.30±0.03 b	

Heart rate (HR)
[1]Reference values according to Menzies-Gow (2015)
a,b: Different letters between groups show significant difference ($p<0.05$, Student t test)

T wave also differed between experimental groups, presenting larger values in CG. T wave might be correlated to hyperkalemia or myocarditis, but only when extreme values (>1.4mV) are seen, which was not the case on the present study (Raekallio et al., 1992; Diniz et al., 2011).

ECG has been used to detect myocardial injuries, but biochemical tests are also available, such as the creatine kinase myocardial band (CK-MB) (Argiroudis et al., 1982). IG presented activities of both CK and CK-MB elevated above the reference values for the species. Since CK-MB is a fraction of total CK, CK increase might be correlated solely to CK-MB increase, due to the extreme high activities of CK-MB here presented (Table 9).

Table 9. Values of CK and CK-MB in horses who have undergone several immunization protocols for loxoscelic antivenom production (mean ± SEM).

Parameter	Immunized horses	Reference values for horses[1]
CK (U/L)	386.4±65.2	90.0-270.0
CK-MB (U/L)	318.2±60.5	5.0-25.0

Creatine kinase (CK); creatine kinase (CK); creatine kinase/isoenzyme MB fraction (CK-MB)
[1]Reference values according to Laboratory of Clinical Analyses, Veterinary Hospital, UFMG.

Increase in CK-MB activities has been previously reported. DiFilippo et al. (2017) reported an increase in CK-MB in healthy Mangalarga Marchador horses after exercise, indicative of myocardial cell injury. Authors then highlighted that this finding could be related to an adaptation to physical exercise, but other variables, such as electrolyte imbalance should also be taken in consideration, corroborating with Michima et al. (2010). Myocardial hypoxia is one of the hypotheses that can lead to myocardial injury and thus, increase in CK-MB activities (Piper et al., 1984; Tharwat et al., 2013). Some studies, however, have proven that increase in CK-MB do not reflect in a histological lesion, and comes from a transitory increase of sarcolemma permeability (Lazarim et al., 2007; Teixeira-Neto, 2008). In the present study, it is possible that commercial bleedings might cause a transient ischemia, and thus cause a myocardial injury reflected in ECG alterations and CK-MB elevation. Besides bleeding, aspects regarding *Loxosceles* venom composition could exert a cardiotoxicity as described by Dias-Lopes et al. (2016). Authors have shown impairment of cardiac function due to calcium flow disruption and its increase in intracellular concentration, which might explain the high level of rhythm abnormalities in ECG recordings of the present study as well as increase in CK-MB activities.

4. Conclusions

Horses that have undergone several complete loxoscelic immunization protocols have kept its erythrocyte-related parameters within inferior reference values for the species, which could be due to age and partake in successive bleeding procedures. No kidney dysfunction was diagnosed and liver-related enzymes were higher when compared to CG, however keeping within reference values. ECG recordings showed some abnormalities and pathological features, showing that the cardiovascular system is the most impaired

during loxoscelic antivenom production, corroborating with thrombophlebitis findings and increased CK-MB activities.

CHAPTER 5

Immunization protocol in rabbits using *Loxosceles* spp. venom

Abstract

Loxoscelism is a severe syndrome caused by spider bite from *Loxosceles* spp. Rabbits are one of the main experimental models used for better understanding of loxoscelic venom systemic and local effects. Eleven male New Zealand rabbits were used. Five were allocated as a control group (CG), that received adjuvant (montanide) and phosphate-buffer saline. The six remaining rabbits received 21µg of *Loxosceles* spp. venom using montanide as adjuvant (VG). After five immunization cycles, a trial with 7µg of *L. intermedia* venom was performed, and dermonecrotic lesions were measured. Rabbits were then euthanized, and their organs harvested for histopathology analysis. No erythrocyte-related parameter showed significance, but white blood cell count was higher in VG, corroborating with venom's neutrophil chemotaxis. Liver and kidney functions were also preserved according to blood biochemical panel. ECG showed no alteration between experimental groups and evaluation periods. Histopathology showed a tendency for loxoscelic venom to produce direct and indirect cardiotoxicity, renal toxicity, and hepatotoxicity.

1. Introduction

Spider bites from specimens of the *Loxosceles* genus are responsible for the development of Loxoscelism, a syndrome consisting mainly of dermonecrotic lesions, but that can also lead to fever, renal failure and several hematologic disturbances (Futrell, 1992; Swanson and Vetter, 2006). Popularly known as "brown spiders", their venom consists of a complex mixture of toxins enriched by low molecular mass peptides (Senff-Ribeiro et al., 2008), which include phospholipases, hyaluronidases, serine proteases, metalloproteases, and venom allergens (Futrell, 1992; Oliveira et al., 2005; Gremski et al., 2014; Oliveira-Lima et al., 2016; Langenegger et al., 2019).

Several animal models are used in order to study loxoscelic venom toxic effects, ranging from murine (Oliveira-Lima et al., 2016; Plenge-Tellechea et al., 2019) to rabbits (Mangili et al., 2003; Tavares et al., 2004; Tavares et al., 2011). A multitude of injuries can be inducted by loxoscelic venom, such as platelet aggregation (Tavares et al., 2011), acute kidney injury (Lucato et al., 2011), cardiotoxicity (Dias-Lopes et al., 2010), and even brain damage (Plenge-Tellechea et al., 2019). However, little is known about overall

impacts on the health status of these animals, during and after venom contact, which could aid in better understanding venom dynamics.

Therefore, the aim of this study is to evaluate loxoscelic venom toxic effects in rabbits that have undergone *Loxosceles* spp. venom immunization protocol, through clinical, hematological, electrocardiographical, and histopathological evaluations of these animals.

2. Material and methods

2.1 Ethical statement

All procedures involving rabbits were conducted according to animal welfare guidelines and the approval of the Ethical Committee for the Use of Animals of the Federal University of Minas Gerais (CEUA/UFMG), under protocol number 388/2017 (Annex III).

2.2 Rabbits, venom and immunization protocol

Eleven male New Zealand rabbits (*Oryctolagus cuniculus*), weighing approximately 2.8 kg, were kept under controlled conditions at the Federal University of Minas Gerais. Rabbits were kept in individual cages and fed twice a day with commercial ration and water *ad libitum*, being closely monitored for any relevant clinical alteration. Five rabbits composed the control group (CG) and six rabbits, the venom group (VG). Animals were immunized and sampled for blood. Electrocardiographic (ECG) recordings were also performed. Immunization protocol and sampling moments are described in Table 10.

Samples from *L. gaucho*, *L. intermedia* and *L. laeta* venom were gently provided by Dr. João Carlos Minozzo (CPPI, PR), following the same extraction guidelines previously mentioned for use in horses' immunization in Chapters 3 and 4. Lyophilized venom was kept on -20°C, in the dark, until its use. Venom proteins' electrophoretic pattern was the same as previously shown in Fig. 3 (Chapter 3).

CG received 1 mL of montanide adjuvant + 1 mL of phosphate-buffer saline (PBS), whereas VG received 7µg of *L. intermedia*, 7µg of *L. laeta* and 7µg of *L. gaucho* venoms diluted in 973.75µL of PBS + 1 mL of montanide. A total of five immunizations were

performed. On day 68 of the immunization cycle, rabbits from both experimental groups underwent a trial in which a minimum necrotizing dose (MND) of *L. intermedia* was inoculated on their ear. A 7µg MND was calculated according to Furlanetto (1961) methodology, and dermal lesions were measured using a ruler as well as ImageJ® software. One rabbit of each experimental group did not undergo this trial. On day 75, rabbits were euthanized with 100mg/Kg of thiopental intravenously, according to guidelines established by the Brazilian Guide of Good Practices in Euthanasia (CFMV, 2013).

Table 10. Immunization protocol of rabbits from VG using *Loxosceles* spp. venom.

Immunization status	Day of the cycle	Total Venom amount	Venom amount per species of *L. intermedia, L. laeta* and *L. gaucho*	PBS	Montanide
T0	0	Clinical examination, blood sampling, ECG recordings			
T1	1	21 µg	7 µg	973.75 µL	1 mL
T2	15	21 µg	7 µg	973.75 µL	1 mL
T3	16	Clinical examination, blood sampling, ECG recordings			
T4	30	21 µg	7 µg	973.75 µL	1 mL
T5	45	21 µg	7 µg	973.75 µL	1 mL
T6	46	Clinical examination, blood sampling, ECG recordings			
T7	60	21 µg	7 µg	973.75 µL	1 mL
T8	62	Blood sampling for CBC, ECG recording			
T9	68	MND trial using *L. intermedia* venom			
T10	75	Euthanasia			

CBC: complete blood cell count; MND: minimum necrotizing dose

2.3Hematological evaluation

Rabbits were physically restrained using a towel. No sedation was needed. Blood sampling was performed after the antisepsis of the ear's marginal vein region using 70% alcohol. Ethylenediaminetetraacetic acid (EDTA) and clot activator tubes were used (*BD Vacutainer® - Becton Dickinson*) in order to perform hematologic and biochemical analyses, respectively. Hematimetric indexes were obtained using an automatic cell counter (*pocH-100Iv-Diff®*); serum biochemistry was analyzed by an automatized biochemistry equipment (*Cobas Mira Plus®*) under clinical pathologist supervision.

Blood parameters evaluated were as follows: red blood cell count (RBC); packed cell volume (PCV); mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC); white blood cell count (WBC); red blood cell distribution width (RDW); lymphocytes, and sum of other WBC, such as neutrophils, monocytes and basophils (OTH); total platelet count (PLT); mean platelet volume (MPV); platelet distribution width (PDW); platelet clump (P-LCR); urea; creatinine; alanine aminotransferase (ALT); aspartate transaminase (AST); alkaline phosphatase (ALP); gamma-glutamyl transpeptidase (GGT); glucose; amylase; total proteins (TP); albumin; globulins; cholesterol; triglyceride and lactate.

2.4Electrocardiographic evaluation

Rabbits underwent an ECG evaluation using a portable 12-channel digital electrocardiograph (*TEB ECG Vet® - Tecnologia Eletrônica Brasileira S.A.*). ECG recordings were acquired in a quiet environment and with rabbits in lateral horizontal decubitus position. Recordings were made at 25 mm/sec speed and sensitivity of 1 cm = 1 mV. Bipolar (DI, DII, DIII) and augmented unipolar (aVR, aVL, aVF) leads were recorded. The following parameters were evaluated: cardiac rhythm; HR; P (ms); P (mV); PR, QRS, and QT intervals; R and T waves and ST segment levels. The cardiac axis was calculated according to Tilley (1992).

2.5 Histopathology

After euthanasia, an immediate necropsy was performed, and significant fragments of liver, kidney, spleen, heart, lungs, and skin were collected for microscopic evaluation. They were fixated in 10% formaldehyde and afterward were embedded in paraffin. Histological sections were obtained with 4μm thickness and dyed using Hematoxylin and Eosin (HE) and Periodic Acid Schiff (P.A.S.) techniques. All samples were processed in the Laboratory of Pathology of UFMG Veterinary School.

2.6 ELISA

MaxiSorp plates purchased from NUNC were coated overnight at 4°C with 100 μL of a 5 μg/mL solution of *L. intermedia, L. gaucho,* and *L. laeta* venoms in 0.02 M sodium bicarbonate buffer, pH 9.6. After blocking [1% skimmed non-fat milk in phosphate buffer saline (PBS)] and washing (0.05% Tween 20-PBS), sera from T0 and immune rabbits

were added in serial dilution from 1/400 to 1/256,000 and incubated for 1 h at 37°C. Plates were washed and incubated with anti-rabbit IgG conjugated with horseradish peroxidase (HRP, Sigma-Aldrich A9292) diluted 1/4,000, for 1 h at 37°C. ELISA was carried out as described by Chavez-Olórtegui et al. (1991). Absorbance values were determined at 492 nm using an ELISA plate reader (BIO-RAD, 680 models). Duplicate assays were taken for all samples and means calculated.

2.7Statistical analysis

Statistical analysis was carried out using the SAS (version 9.0) software program. The obtained data were statistically analyzed using a mixed linear model approach of SAS (PROC MIXED), using first-order autocorrelation covariate structure. Animals were considered as a random factor, with each animal nested within treatments, with repeated measurements over time. P values < 0.05 were considered to indicate significance.

3 Results and discussion

No relevant clinical alteration was noted during immunizations, except a slight soreness on inoculation sites in some animals. After trial with DMN, however, necrotic lesions were observed on the rabbits' ear. Rabbits from VG (Fig 11A) presented significant smaller dermal lesions (≈ 0.08 cm^2 of lesion with 0.01 cm^2 of necrosis) than those of CG (≈ 1.08 cm^2 of lesion with 0.11 cm^2 of necrosis) (Fig 11B), proving that not only an adequate sera conversion was achieved, but that neutralizing antibodies were successfully produced, as was also shown in ELISA assay (Fig. 12).

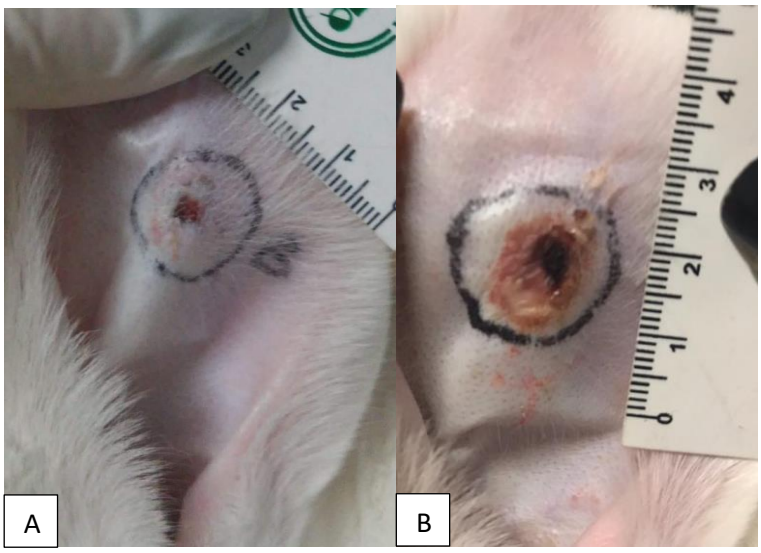

Fig. 11 Measurement of dermonecrotic lesions after trial using a 7µg MND of L. intermedia in rabbits that have undergone immunization protocol using venom from Loxosceles spp. (A) Venom group showing a minimal lesion with absence of necrosis. (B) Control group showing a larger lesion area accompanied by necrosis.

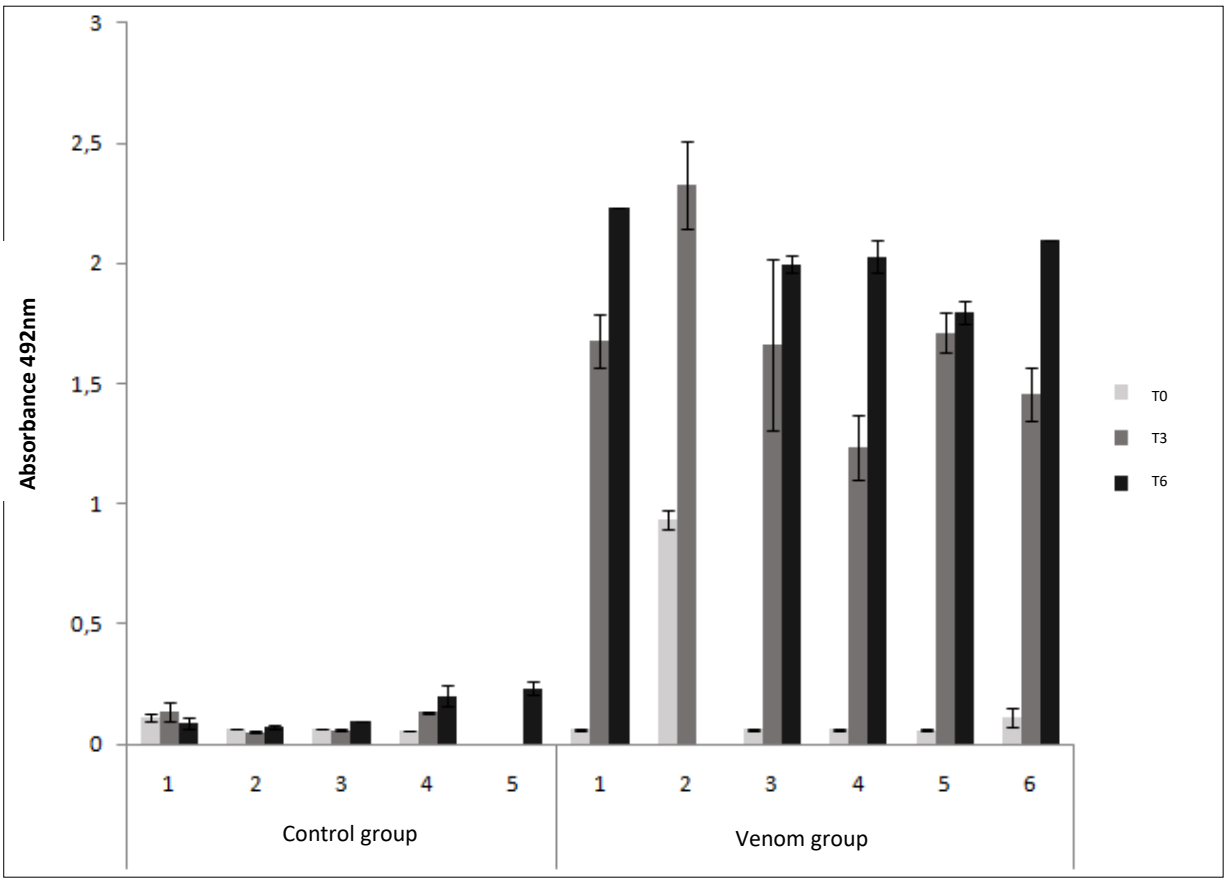

Fig. 12 Immunoreactivity of rabbits immunized with *Loxosceles* spp. venom by ELISA in T0, T3 and T6. Plates were coated with a 5 µg/ml of each venom (*L. intermedia, L. gaucho*, and *L. laeta*) and sera was tested in 1/200 dilution and revealed with rabbit anti-IgG 1/5000 and o-phenylediamine dihydrochloride). Control group received montanide and PBS and venom group 21 µg of *Loxosceles* spp. venom and montanide. The absorbance of samples was determined at 492nm.

Rabbits were evaluated regarding their hematological and biochemical panels in different sampling moments. Results are described in Tables 11 and 12.

Table 11. Complete blood count panel of rabbits that underwent an immunization protocol using *Loxosceles* spp. venom + montanide (Venom group) and of rabbits that received montanide + PBS (Control group).

Parameter		Control group	Venom group	Reference values for rabbits[1]
WBC (cell x 10^3/µL)				5.2-12.5
	T0	8.25±1.16 b	10.3±1.37 c	
	T3	8.28±1.13 b,B	13.5±0.62 b,A	
	T6	12.1±0.61 a	14.8±0.32 a,b	
	T8	11.6±1.32 a,B	16.7±1.16 a,A	
RBC (cell x 10^6/µL)				5.4-7.6
	T0	6.59±0.17 a	6.66±0.07	
	T3	6.31±0.13 b	6.54±0.08	
	T6	6.63±0.15 a	6.62±0.07	
	T8	6.61±0.13 a	6.77±0.02	
HGB (g/dL)				10.0-17.4
	T0	13.9±0.36 a	13.5±0.22 a	
	T3	13.0±0.37 b	13.4±0.32 a	
	T6	14.3±0.37 a	13.8±0.11 a	
	T8	6.30±0.50 c	6.60±0.25 b	
PCV (%)				33.0-50.0
	T0	42.6±0.78	42.2±0.57	
	T3	41.9±0.86	42.1±0.51	
	T6	42.9±0.62	42.2±0.66	
	T8	43.2±0.94	43.7±1.11	
MCV (fL)				60.0-69.0
	T0	64.8±0.79 b	63.4±0.69	
	T3	66.4±1.07 a	64.5±0.98	
	T6	64.8±1.01 b	63.8±0.78	
	T8	65.3±0.99 a,b	64.5±1.49	
MCH (pg)				19.0-22.0
	T0	21.1±0.24 a	20.3±0.33 a	
	T3	20.7±0.39 a	20.5±0.54 a	
	T6	21.6±0.22 a	20.9±0.32 a	
	T8	9.53±0.62 b	9.75±0.34 b	
MCHC (g/dL)				30-35%
	T0	32.6±0.27 a	32.1±0.18 a	
	T3	31.1±0.44 b	31.8±0.67 a	
	T6	33.4±0.54 a	32.8±0.48 a	
	T8	14.6±0.90 c	15.1±0.21 b	
PLT (cell x 10^3/µL)				250.0-650.0
	T0	184.7±37.8 b	277.8±12.3 a,b	
	T3	147.0±39.7 b	185.4±32.4 b	
	T6	341.3±41.1 a	324.2±24.4 a	
	T8	263.7±63.0 a,b	251.3±21.5 a,b	
Lymphocytes (%)				30.0-85.0
	T0	62.5±4.45	65.2±3.30 a	
	T3	60.6±5.64 A	45.9±3.38 b,B	
	T6	53.0±2.94	49.4±3.55 b	
	T8	53.0±1.90	47.2±2.19 b	
OTHR (%)				
	T0	28.2±3.35	28.2±2.70 b	Neutrophils 20.0-75.0
	T3	32.5±4.94 B	46.0±3.55 a,A	Basophils 1.0-7.0
	T6	33.7±3.33	43.0±2.25 a	Monocytes 1.0-4.0
	T8	36.3±4.65	44.2±2.18 a	
Eosinophils (%)		17.5±4.13 a	13.2±4.14	1.0-4.0
	T0	9.73±2.89 b	8.12±0.90	

	T3	18.3±2.79 a,A	7.64±1.98 B
	T6	10.7±3.06 a,b	8.65±0.36
	T8		
Lymphocytes (cell x 10^3/μL)			
	T0	6.15±1.25 a,b	7.80±1.70
	T3	4.94±0.83 b	6.24±0.63
	T6	7.00±0.25 a	7.34±0.63
	T8	6.20±0.84 a,b	7.83±0.54
OTHR (cell x 10^3/μL)			
	T0	2.65±0.05	3.30±0.20 b
	T3	2.82±0.72 B	6.20±0.47 a,A
	T6	4.50±0.50 B	6.32±0.24 a,A
	T8	4.23±0.69 B	7.38±0.75 a,A
Eosinophils (cell x 10^3/μL)			
	T0	1.55±0.47 a,b	1.25±0.39
	T3	0.80±0.25 b	1.10±0.12
	T6	2.17±0.28 a,B	1.12±0.29 A
	T8	1.20±0.32 a,b	1.45±0.12
RDW-SD (fL)			
	T0	34.9±0.60 a	35.4±0.55 b,c
	T3	36.1±1.02 a	37.0±0.85 a
	T6	32.7±0.61 b	34.2±0.65 b
	T8	35.0±1.13 a	36.3±0.89 a,c
RDW-CV (%)			
	T0	12.3±0.44 a,b	13.3±0.77 a,b
	T3	13.0±0.93 a	14.0±0.61 a
	T6	11.0±0.60 b	11.8±0.40 b
	T8	12.2±1.18 a,b	14.0±0.42 a
PDW (fL)			
	T0	8.12±0.55 b	8.10±0.39 b
	T3	9.17±0.50 a	9.78±0.62 a
	T6	6.88±0.32 c	7.44±0.40 b
	T8	6.40±0.30 c	7.05±0.37 b
MPV (fL)			
	T0	7.94±0.32 b	8.03±0.28 b
	T3	8.68±0.29 a	8.94±0.33 a
	T6	7.15±0.26 c	7.52±0.23 b,c
	T8	6.83±0.15 c	7.35±0.30 c
P-LCR (%)			
	T0	9.00±2.01 b	9.23±1.91 b
	T3	14.3±2.55 a	14.7±2.80 a
	T6	5.23±1.05 b	6.66±0.95 b
	T8	4.53±0.54 b	5.93±1.09 b

White blood cell count (WBC); red blood cell count (RBC); packed cell volume (PCV); mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC); red blood cell distribution width (RDW); sum of other WBC, such as neutrophils, monocytes and basophils (OTHR); total platelet count (PLT); platelet distribution width (PDW); mean platelet volume (MPV); platelet clump (P-LCR).
[1]Reference values according to (Archetti et al., 2008).
a,b,c Statistical difference (mixed linear model, $p<0.05$) between experimental moments within groups
A,B Statistical difference (mixed linear model, $p<0.05$) between experimental groups in the same sampling moment

No significant difference was seen regarding erythron parameters. PCV, RBC, PCV, MCV, MCH, and MCHC were kept within reference values for the species and suffered minimal variation between sampling times and experimental groups. Platelet-related parameters followed this pattern as well. A significant difference was observed, however, within experimental groups and sampling moments regarding WBC. In both experimental groups, a pattern of increase in WBC was observed after T0. Since both groups received adjuvant (montanide), it is important to highlight that they increase antigen uptake by

antigen-presenting cells (APCs), activate or aid in maturing APCs (e.g., dendritic cells), besides inducing the production of immunoregulatory cytokines, activating inflammasomes and inducing local inflammation and cellular recruitment (Apostólico et al., 2016). Therefore, both groups presented an inflammatory pattern of WBC increase, but only VG kept these values above reference ranges for the species and were significant different from CG in T3 and T9. This difference might be attributed to venom action. Phospholipase-D, the main toxic factor in *Loxosceles* spp. venom, is the primary inductor of both expression and secretion of inflammatory mediators, especially in fibroblasts. This induction culminates in fibroblast secreting inflammatory mediators that will contribute to monocyte recruitment to the site of damage, such as IL-6, IL-8, CXCL1/GRO-α, and CCL2/MCP-1 (Rojas et al., 2017). A decrease in lymphocytes, accompanied by an increase in neutrophils and monocytes was observed on both groups during all sampling periods and were statistically significant between VG and CG, with VG presenting higher values. This result was already expected, since neutrophils are largely recruited in acute inflammation (Dodds, 1997). An interesting finding, however, was regarding eosinophils. Both groups kept eosinophil values way above reference values for the species. *Loxosceles* spp. venom has a TCTP toxin, with a histaminergic effect related to pro-inflammatory properties, acting as an allergen (Boia-Ferreira et al., 2019). Allergic reactions are accounted for eosinophilia, which might explain results from VG, but not from CG. A plausible explanation is that rabbits' neutrophils have eosinophilic granules, which can make them be mistaken by eosinophils (Archetti et al., 2008). Thus, a more pronounced neutrophilia might be happening in both experimental groups and was underestimated due to the eosinophil overestimated count. However, eosinophilia was observed in histopathology analysis of some organs described later in this section.

Table 12. Biochemical panel of rabbits that underwent immunization protocol with *Loxosceles* spp. venom + montanide (Venom group) and rabbits that received montanide + PBS (Control Group) (mean ± SEM).

Parameter		Control Group	Venom group	Reference values for rabbits[2]
Urea (mg/dL)				2.0-8.5
	T0	44.0±1.01	42.3±1.80	
	T3	42.1±2.74	33.9±2.71	
	T6	42.7±9.15	33.9±2.84	
Creatinine (mg/dL)				0.5-2.6
	T0	1.52±0.06	1.64±0.10	
	T3	1.63±0.25	1.42±0.21	
	T6	1.51±0.14	1.43±0.13	
ALT (U/L)				25.0-65.0
	T0	33.0±6.92 a	27.7±3.32 a	
	T3	29.2±5.18 a,B	15.0±1.60 b,A	
	T6	13.4±1.03 b	13.8±2.70 b	
AST (U/L)				20.0-120.0
	T0	31.6±6.35	27.2±3.82	
	T3	27.0±4.62	18.3±5.87	
	T6	17.0±1.80	21.3±4.12	
ALP (U/L)				10.0-86.0
	T0	56.2±4.89	83.9±17.0	
	T3	48.7±5.54	63.0±10.6	
	T6	33.0±8.58	51.8±10.7	
GGT (U/L)				10.0-98.0
	T0	14.3±2.55 b	13.4±1.85	
	T3	40.2±7.70 a,A	14.3±3.55 B	
	T6	16.4±1.21 b	16.8±1.90	
Glucose (mg/dL)				74.0-148.0
	T0	94.2±3.09 a	103.3±6.23 b	
	T3	60.7±9.03 b,B	102.7±10.2 b,A	
	T6	105.2±4.03 a,B	139.0±18.2 a,A	
Amylase (U/L)				200.0-500.0
	T0	409.0±28.2	329.4±86.7	
	T3	328.8±20.0	347.5±25.3	
	T6	322.7±73.4	295.0±22.6	
TP (g/dL)				5.0-7.5
	T0	6.10±0.30 B	6.87±0.14 a,A	
	T3	6.09±0.19	5.93±0.20 b	
	T6	5.80±0.08	6.04±0.21 b	
Albumin (g/dL)				2.7-5.0
	T0	3.61±0.40 a,b	3.88±0.15	
	T3	2.91±0.24 b,B	3.75±0.11 A	
	T6	3.76±0.09 a	3.80±0.19	
Globulines (g/dL)				1.5-2.7
	T0	2.49±0.11 b,B	2.98±0.10 a,A	
	T3	3.18±0.13 a,A	2.17±0.25 b,B	
	T6	2.04±0.17 c	2.24±0.09 a	
Cholesterol (mg/dL)				10.0-100.0
	T0	31.2±2.29	31.2±3.18	
	T3	42.5±3.85 A	30.0±3.82 B	
	T6	42.3±5.43 A	32.4±3.34 B	
Triglycerides (mg/dL)				50.0-200.0
	T0	73.1±5.47	75.5±8.77	
	T3	71.1±13.3	101.1±24.3	
	T6	79.5±27.16	72.7±8.45	
Lactate (mg/dL)				8.11 to 21.2
	T0	89.2±19.8	106.5±25.2	
	T3	92.9±23.4	54.6±8.83	
	T6	52.8±4.09	69.2±9.11	

Alanine aminotransferase (ALT); aspartate transaminase (AST); alkaline phosphatase (ALP); gamma-glutamyl transpeptidase (GGT); total protein (TP).

[2]Reference values according to Suckow et al. (2012)

a,b,c Statistical difference (mixed linear model, $p<0.05$) between experimental moments within groups

A,B Statistical difference (mixed linear model, $p<0.05$) between experimental groups in the same sampling moment

Rabbits kept urea levels over the reference ranges for the species, probably due to the high protein content of their diets, since no statistical difference was observed within sampling moments and experimental groups. Creatinine levels, however, were kept within reference values. The influence of diet composition in rabbit's hematological and blood biochemical parameters has been previously reported (Etim et al., 2014; Gugołek et al., 2018). Rabbits should receive a 92% hay and vegetables diet, but in experimental conditions, unfortunately, this was not doable, and pellets composed the whole of their diet. This diet composition might as well explain why lactate levels were kept above reference ranges but with no statistical difference between sampling moments and experimental groups. Due to their diet composition, a minor acidosis should explain the values of this variable as well as glucose levels. Glucose kept an increasing pattern during experimental periods, with statistical significance between VG and CG after T0. However, these levels were always kept within reference values for the species. Increased glucose might be related to a high-sugar diet, but also due to lack of exercise. Rabbits were confined in individual cages and had limited access to exercise themselves vigorously. Stress and pain can also be hyperglycemic factors (Candasamy et al., 2014). Rabbits on the present study needed to be handled for sampling, which could have caused stress, and manipulation could trigger a pain response in already sore injection sites. Differences between VG and CG, with VG with significant glucose levels, might also be explained by venom action, since inflammation is responsible for glucose increase as well (DeFranco et al., 2007).

Determining different enzymatic activities in blood can offer valuable information regarding liver lesions, such as the ones caused by drugs, toxins, or infections (Coox and Nelson, 2006). The activities of all liver-related enzymes in the present study, such as GGT, ALT, AST, and ALP, were kept within reference values for the species. TP, albumin, and globulins levels were kept within reference values for the species as well, corroborating with liver integrity previously mentioned. Apparently, there was not a demand high enough for globulin synthesis, which kept all protein-related variables within the reference range. Perhaps, if more immunizations have had occurred, this could be different, since immunoglobulin production would have been optimized. However, a slight difference regarding cholesterol levels between experimental groups might infer a slight liver impairment in VG. Cholesterol is synthesized in the liver (Guyton and Hall, 2015), and VG presented smaller values of cholesterol than CG, which could imply liver

malfunction, however insidious. Venom itself might be responsible for this, since *Loxosceles* spp. venom does possess a direct hepatotoxic effect (Christoff, 2008).

Due to the venoms' cardiotoxicity (Dias-Lopes et al., 2016), ECG recordings were made on four occasions. Rabbits kept a normal sinus rhythm at all times (Fig. 13). ECG data is shown in Table 13. No major alteration was observed between experimental groups, and evaluation periods and variables were kept within reference ranges for the species. Similarly to what was highlighted regarding TP and globulin values, it could be possible that no arrhythmia was diagnosed because there were not enough immunizations performed able to promote a cardiotoxic effect to become distinct in ECG, differently from what was observed in horses from Chapters 3 and 4. Their prolonged immunization periods reflected in more clear cardiac alterations, as well as those regarding protein synthesis parameters.

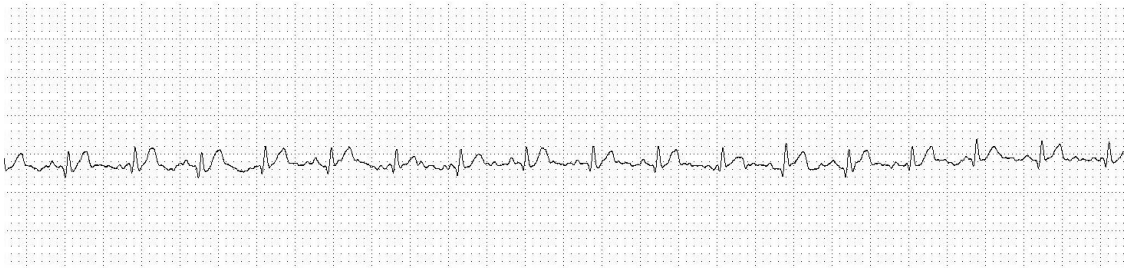

Fig. 13. Electrocardiographic recording of a rabbit in T0 presenting normal sinus rhythm.

Table 13. ECG recordings of rabbits that underwent immunization protocols with Loxosceles spp. + montanide (Venom group) and rabbits that received montanide + PBS (Control group).

Parameter		Control group	Venom group	Reference values for rabbits[3]
Heart rate (bpm)				198.0-330.0
	T0	196.2±13.11 a,b	226.3±8.86 a	
	T3	199.0±8.42 b	210.5±10.85 b	
	T6	179.3±6.63 a	210.4±18.95 b	
	T8	192.9±9.75 a,b	220.4±18.99 a,b	
P (ms)				10.0-50.0
	T0	35.3±2.64 b	39.4±2.95	
	T3	38.3±2.49 a,b	39.2±3.10	
	T6	37.9±2.22 a,b	41.8±2.51	
	T8	47.2±7.33 a	44.8±1.77	
P (mV)				0.04-0.12
	T0	0.04±0 b	0.03±0.01	
	T3	0.05±0.01 a,b	0.04±0.01	
	T6	0.03±0 b	0.04±0.01	
	T8	0.06±0.01 a	0.05±0	
PR (ms)				40.0-80.0
	T0	55.2±9.09	67.4±9.58	
	T3	57.5±8.07	56.5±7.41	
	T6	69.6±8.06	66.0±5.32	
	T8	68.3±7.68	75.1±1.94	
QRS (ms)				20.0-60.0
	T0	47.4±4.34	46.5±2.88	
	T3	40.3±2.14	43.3±4.46	
	T6	46.3±4.41	44.0±4.25	
	T8	40.6±3.56	45.7±3.02	
R (mV)				0.03-0.39
	T0	0.12±0.07	0.14±0.05	
	T3	0.11±0.08	0.15±0.02	
	T6	0.04±0.06	0.11±0.01	
	T8	0.06±0.11	0.12±0.03	
QT (ms)				80.0-160.0
	T0	131.6±14.5 b	127.8±6.79	
	T3	165.8±13.5 a	139.5±10.5	
	T6	142.2±8.10 a,b	122.6±17.9	
	T8	145.8±2.25 a,b	142.1±9.89	
T (mV)				0.05-0.17
	T0	0.16±0.04	0.12±0.08	
	T3	0.11±0.03	0.05±0.02	
	T6	0.07±0.01	0.06±0.02	
	T8	0.06±0.01	0.08±0.02	

[3]Reference values according to Lord et al., 2010

a,b,c Statistical difference (mixed linear model, $p<0.05$) between experimental moments within groups

A,B Statistical difference (mixed linear model, $p<0.05$) between experimental groups in the same sampling moment

Histology analysis was also performed, and main alterations are shown in Figs. 14 to 23.

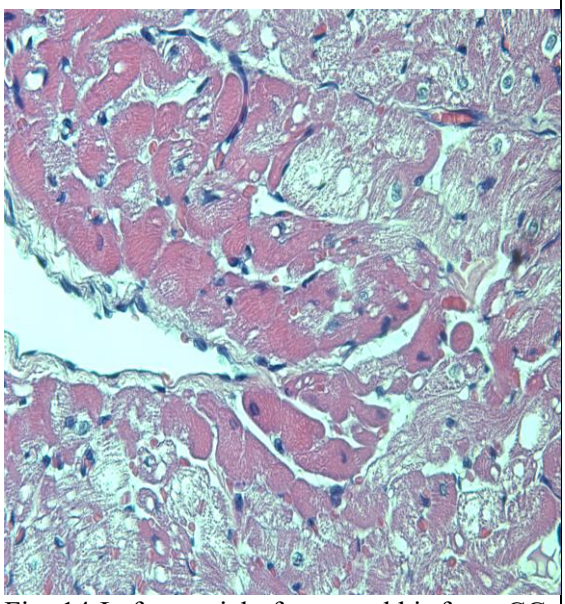

Fig. 14 Left ventricle from a rabbit from CG showing mild cardiomyocyte degeneration. This rabbit did not partake the trial period, having no contact with venom, only montanide adjuvant (HE, 400X). Glycogen granules were seen predominantly in the cytoplasm of cardiomyocytes and mildly in blood vessels when stained with P.A.S.

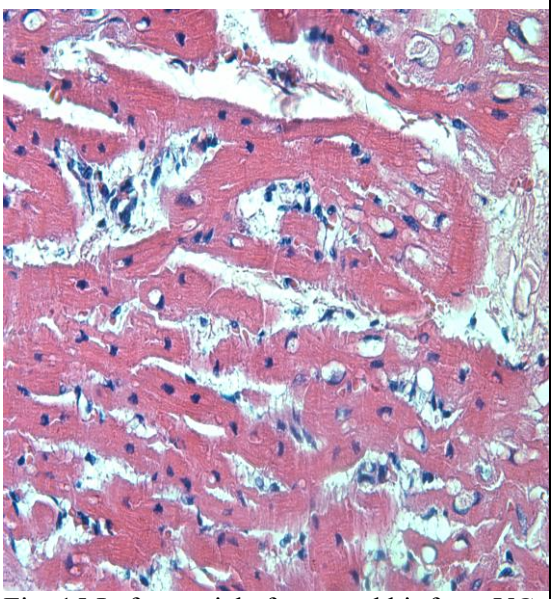

Fig. 15 Left ventricle from a rabbit from VG showing severe and diffuse congestion, with lymph-histiocytic infiltrate near blood vessels. Discrete multifocal cardiomyocyte degeneration was also observed. This rabbit did not partake the trial period and was inoculated with venom only accompanied by adjuvant (HE, 400X). P.A.S. stain was not positive.

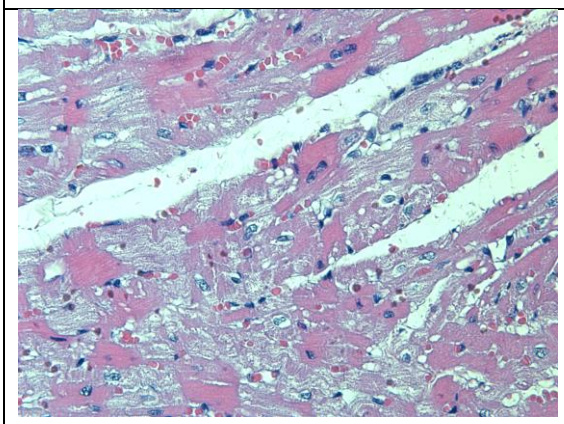

Fig. 16 Left ventricle from a rabbit from CG.] showing hypereosinophilic stain, loss of cardiac striation and cardiomyocyte degeneration, as well as moderate hemorrhage in multiple focal areas. Despite being in CG, this rabbit received a 7µg of *L. intermedia* venom during the trial period, which could explain the aforementioned alterations (HE, 400X). Cardiomyocytes cytoplasm were also stained by P.A.S.

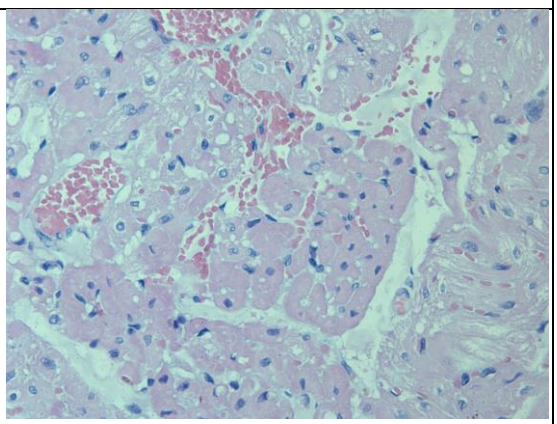

Fig. 17 Left ventricle from a rabbit from VG showing severe and diffuse congestion, with multifocal hemorrhage areas. Multifocal cardiomyocyte degeneration was also observed. Besides undergoing immunization protocols, this rabbit also underwent the trial period receiving a 7µg of *L. intermedia* venom (HE, 400X). P.A.S. stain was not positive.

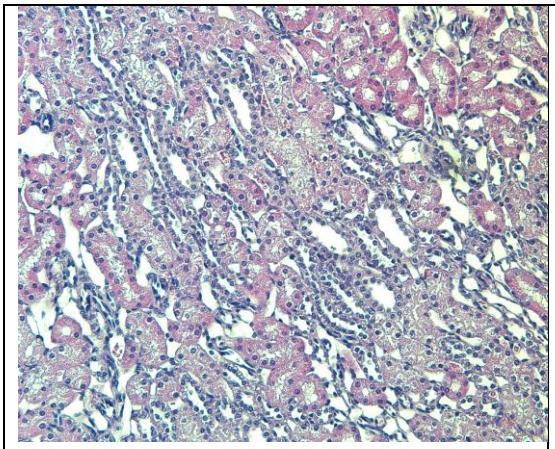

Fig. 18 Kidney cortex from a rabbit from CG showing mild degeneration of the tubular epithelium. This rabbit did not partake the trial period, having no contact with venom, only montanide adjuvant (HE, 400X). A positive stain was seen in the glomeruli (Bowman capsule), blood vessel walls and near renal tubules using P.A.S.

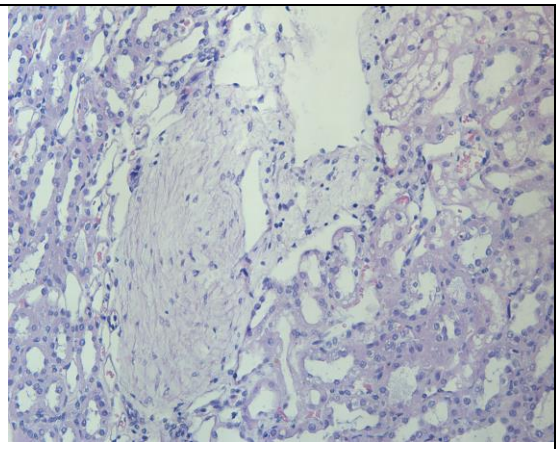

Fig. 19 Kidney from a rabbit from VG showing mild fibrosis of the medullar region with lymph-histiocytic infiltrate. Besides undergoing immunization protocols, this rabbit also partook in the trial period receiving a 7µg of *L. intermedia* venom (HE, 400X). P.A.S. stain was not positive.

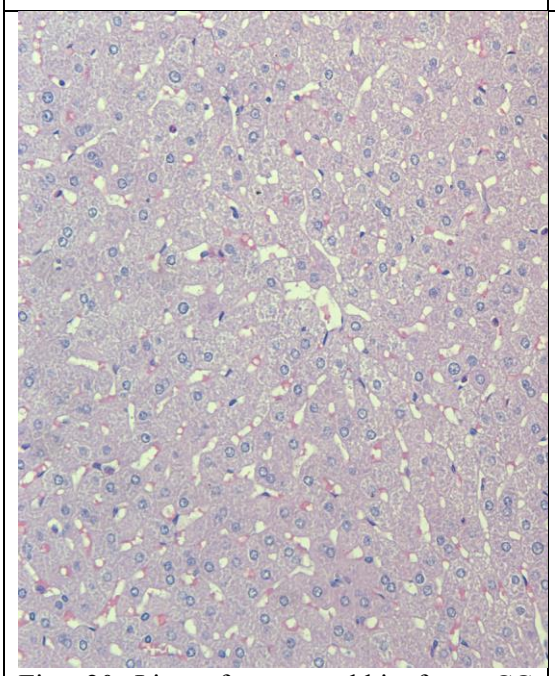

Fig. 20 Liver from a rabbit from CG showing multifocal lymph-histiocytic infiltrate and diffuse glycogenic degeneration. Despite being in CG, this rabbit received a 7µg of *L. intermedia* venom during the trial period, which could explain the aforementioned alterations (HE, 400X). P.A.S. stain positive in hepatocytes.

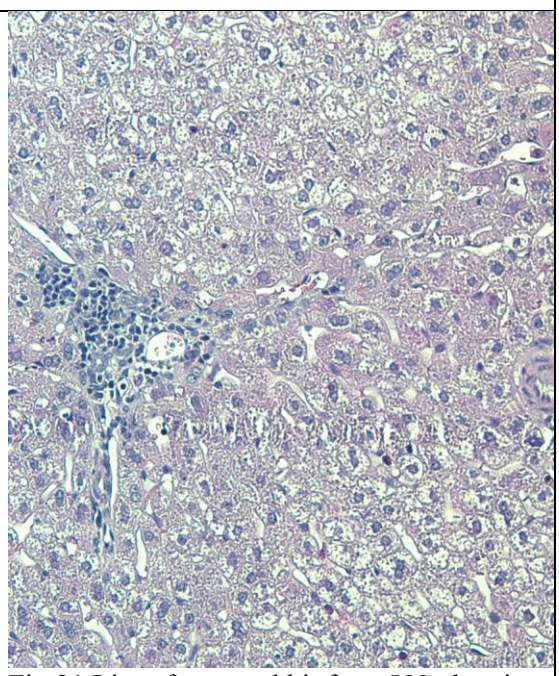

Fig 21 Liver from a rabbit from VG showing multifocal moderate hemorrhagic areas and glycogenic degeneration. Besides undergoing immunization protocols, this rabbit also underwent the trial period receiving a 7µg of *L. intermedia* venom (HE, 400X). P.A.S. stain was intensely positive in hepatocytes and in connective tissues surrounding the portal triad.

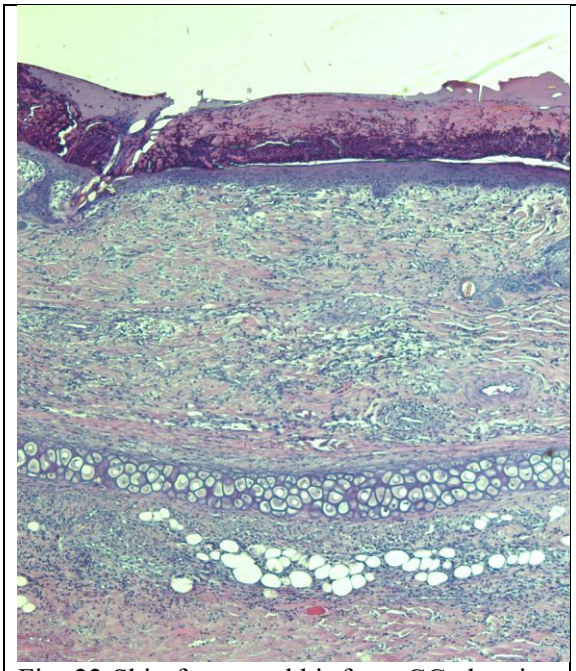

Fig. 22 Skin from a rabbit from CG showing intense necrosis accompanied by cartilage necrosis (HE, 100X).

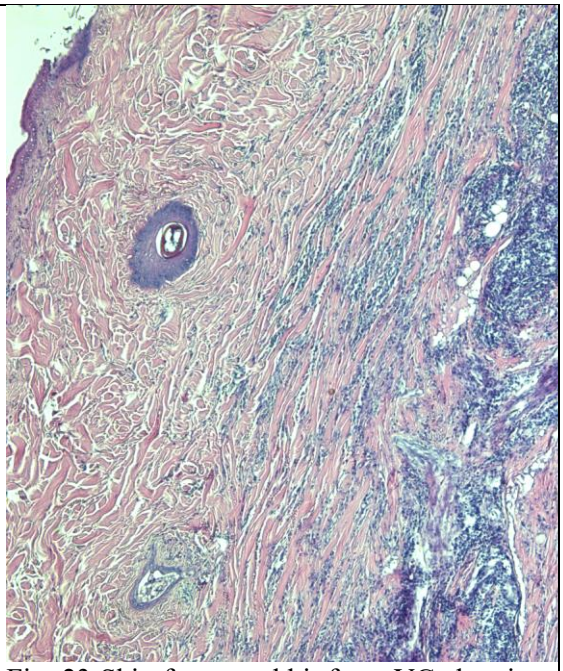

Fig. 23 Skin from a rabbit from VG showing extensive and deep heterophilic lymph-histiocytic infiltrate in the epidermis, accompanied by cartilage mild degeneration (HE, 400X).

Some heart lesions were observed in experimental groups (Figs 14-17). *Loxosceles* spp. venom cardiotoxicity was described earlier by Dias-Lopes et al. (2016). These authors have shown impairment of cardiac function mainly due to disruptions in calcium flow and abnormal increase of its intracellular concentration. Since some mild cardiomyocyte degeneration was diagnosed on CG group, it is feasible to postulate that maybe the adjuvant use (montanide) might be responsible for this effect. Montanide is a water-in-oil emulsion, mainly composed of mineral oil and a surfactant from the mannide monooleate family. Its mode of action is based on enhancing antigen-specific antibody titers and responses coming from cytotoxic T-lymphocyte. A depot theory surrounds montanide mode of action. Depot formation would slowly release antigens at the immunization site. However, other modes of action were also proposed, such as an inflammation promoter (thus stimulating APCs recruitment) and lymphocyte trapping (thus stimulating accumulation of lymphocytes in lymph nodes and optimizing contact with APCs) (Waghmare et al., 2009; Khabazzadeh and Mehdi, 2016; Van Doorn et al., 2016). Cardiomyocyte degeneration, in a more severe degree, was also observed in VG and rabbits in CG that have undergone trial with *L. intermedia* venom. In addition, rabbits that were directly in touch with both montanide and loxoscelic venom presented much more severe cardiac alterations, with lymph-histiocytic infiltrate and hemorrhaging areas, corroborating with the existence of direct venom cardiotoxicity. Plenge-Tellechea et al. (2019) found acanthocytosis (an abnormality of erythrocyte membrane) in rats injected with *Loxosceles apachea* venom, which could be attributed to hemorrhage observed in the present study.

Renal function was not impaired in any rabbit according to serum biochemical panel. Similar to what was observed in the histopathology analysis of the heart, kidneys from rabbits that did not receive the loxoscelic venom presented a mild degeneration, probably due to montanide and its inflammation promotion (Van Doorn et al., 2016). VG group, however, presented fibrosis and moderate lymph-histiocytic infiltrate, inferring a chronic renal impairment. These results corroborate with Lucato et al. (2011), who showed that there was a sudden and significant drop in glomerular filtration rate, renal blood flow, and urinary output, as well as an increase in renal vascular resistance, in animals injected with *Loxosceles gaucho* venom. These authors were able to diagnose acute kidney epithelial tubular cells degenerative changes, cell debris, and detached epithelial cells in

tubular lumen without, however, glomerular or vascular changes. They concluded that these alterations were due to renal vasoconstriction and rhabdomyolysis. A more prolonged experimental period on the present study might justify the more chronic alterations found, such as fibrosis.

Regarding the pathological examination of liver, glycogenic degeneration was the major finding in rabbits that received *Loxosceles* spp. venom. Christoff (2008) showed that *Loxosceles* spp. venom holds a direct hepatotoxic effect, due to direct venom action in degenerating hepatocytes membrane and neutrophil infiltration. Glycogenic degeneration might be due to a decrease in glycogen mobilization, and its accumulation might cause steatosis that can evolve into fibrosis. The latter was not observed in the present study, probably due to insufficient experimental time. These histopathological alterations, however, were not accompanied by increased serum activities of liver-related enzymes, allowing to classify these alterations as mild. It might be possible, though, that this degeneration could reflect in glucose metabolism, which would explain the higher levels of blood glucose observed on rabbits of VG in the present study.

Skin lesions diagnosed in the present study were characteristic of the dermonecrotic lesions previously described in loxoscelic envenomation (Patel et al., 1994; Pauli et al., 2006; Gremski et al., 2014). CG presented more extensive lesions than those of VG group, probably because immunization protocol was able to confer enough protective antibody titers. The pathophysiology of dermonecrosis is not yet totally elucidated. Phospholipase-D is the main responsible for dermonecrosis, promoting neutrophilic infiltration, complement activation, platelet aggregation, edema, and increased vascular permeability (Forrester et al., 1978; Rodríguez e Mendéz, 2008; Chaves-Moreira et al., 2011). Neutrophils' role on the inflammatory response is directly linked to the endothelial dysfunction that will lead to an indirect and dysregulated neutrophil activation (Patel et al., 1994), which, in turn, will cause the up-regulated expression of IL-6, IL-8, CXCL-1, CXCL-2 and MCP-1 (Dragulev et al., 2007; Rojas et al., 2017). Endothelial dysfunction also plays an important role in dermonecrosis, since it occurs in both extracellular matrix and cellular surface, leading to subendothelial vacuoles and fibrin formation, accompanied by morphologic alterations, such as cellular retraction, reduction of intercellular adhesion, and disorganization of actin filaments. These disruptions on endothelial surfaces and cellular adhesion structures act directly on blood vessel stability

and can cause leukocyte and platelet activation, increased vascular permeability, and promote disseminated intravascular coagulation (Paludo et al., 2006). Endothelial dysfunction is a major trigger to platelet aggregation, which will also contribute to dermonecrosis, since dermal venules and arterioles will be occluded, thus contributing to hypoxia and cellular devitalization (Zanetti et al., 2002).

4.Conclusions

Rabbits that have undergone *Loxosceles* spp. venom immunization protocol showed minor clinical disturbances during the experimental period, keeping blood counts and serum biochemical panel without major alterations. These findings were corroborated by the lack of ECG alterations and the minor histopathological alterations observed in key-organs, such as kidneys and liver. Cardiotoxicity, hepatotoxicity, and renal toxicity previously attributed to loxoscelic envenomation were also here described.

Considerações finais

- O protocolo de imunização do veneno loxoscélico causou impacto na saúde dos equinos avaliados, principalmente após o processo de sangria comercial.
- Apesar da redução significativa de RBC, PCV e hemoglobina em T2, os equinos mantiveram funções renal e hepática dentro dos limites fisiológicos para a espécie.
- Devido à queda dramática de PCV observada em T2, sugere-se a reinfusão de papa de hemácias com o intuito de minimizar os efeitos observados.
- O ECG foi capaz de ilustrar algumas alterações de ritmo cardíaco no equino. Porém, seu real impacto clínico ainda permanece incerto.
- A tromboflebite e os abscessos subcutâneos foram os achados clínicos mais relevantes. No entanto, os mesmos são inerentes ao processo de produção de soro, devido às venopunções sistemáticas e uso de adjuvantes para inoculação, respectivamente.
- Equinos que se submeteram a diversos ciclos de imunização completos para produção de soro anti-loxoscélico não apresentaram alterações hepáticas ou renais, similar ao observado em equinos que passaram somente por um ciclo de imunização e coelhos experimentalmente imunizados.
- Os traçados eletrocardiográficos ilustraram algumas anormalidades e características patológicas de ritmo cardíaco, mostrando que o sistema cardiovascular é o mais afetado ao longo dos protocolos de produção de soro, corroborando com as tromboflebites diagnosticadas e o aumento da atividade da enzima CK-MB.
- Coelhos submetidos à protocolos de imunização com veneno de *Loxosceles* spp. mantiveram o hemograma e a bioquímica sérica sem alterações relevantes.
- Cardio/reno e hepatotoxicidade (atribuídas ao envenenamento loxoscélico) foram vistos nos coelhos, apesar de discretas.
- A produção de antiveneno é fundamental para a saúde pública e os equinos são protagonistas nessa cadeia.

Concluding notes

- Loxoscelic immunization protocol has impacted the horses' health, especially after bleeding for commercial antivenom procurement.
- Despite a significant decrease in RBC, PCV, and hemoglobin in T2, horses kept liver and renal functions within physiological levels.
- Dramatic decrease in PCV was observed in T2, and it is suggested a reinfusion of RBC resuspended in order to minimize the clinical effects observed.
- ECG was able to record some arrhythmias in these horses, but their real clinical impact remains unclear.
- Thrombophlebitis and subcutaneous abscesses were the main clinical findings, but are inherent to antivenom procurement management, due to the systematic venipunctures and use of adjuvants at the venom inoculation sites, respectively.
- Horses that have undergone several complete loxoscelic immunization protocols had no kidney or liver disfunction, similar to naïve horses and rabbits.
- ECG recordings showed some abnormalities and pathological features, showing that the cardiovascular system is the most impaired during loxoscelic antivenom production in horses, corroborated with thrombophlebitis findings and increased CK-MB activities.
- Rabbits that have undergone *Loxosceles* spp. immunization protocol kept blood count and serum biochemical panel without major alterations.
- Cardiotoxicity, hepatotoxicity and renal toxicity previously attributed to loxoscelic envenomation were seen in rabbits, however minimal.
- Production of antivenom is paramount for public health, and horses play a definite role in such.

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ANNEX I

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Prezado(a):

Esta é uma mensagem automática do sistema Solicite CEUA que indica mudança na situação de uma solicitação.

Protocolo CEUA: 159/2019
Título do projeto: Estudo da toxicidade do veneno de Loxosceles spp. (aranha-marrom) em equinos soroprodutores
Finalidade: Pesquisa
Pesquisador responsável: Benito Soto Blanco
Unidade: Escola de Veterinaria
Departamento: Departamento de Clinica e Cirurgia Veterinaria

Situação atual: Decisão Final - Aprovado

Aprovado na reunião do dia 01/07/2019. Validade: 01/07/2019 à 30/06/2024

Belo Horizonte, 01/07/2019.

Atenciosamente,

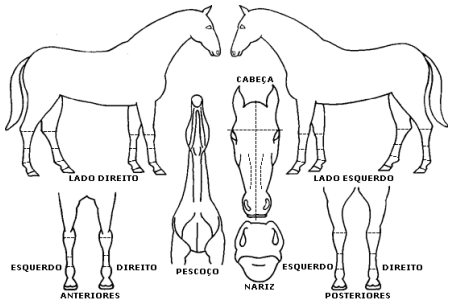
Sistema Solicite CEUA UFMG
https://aplicativos.ufmg.br/solicite_ceua/

Universidade Federal de Minas Gerais
Avenida Antônio Carlos, 6627 – Campus Pampulha
Unidade Administrativa II – 2º Andar, Sala 2005
31270-901 – Belo Horizonte, MG – Brasil
Telefone: (31) 3409-4516
www.ufmg.br/bioetica/ceua - cetea@prpq.ufmg.br

ANNEX II

CENTRO DE PRODUÇÃO E PESQUISA DE IMUNOBIOLÓGICOS

Data: _____
Hora: _____
Identificação: M ☐ F ☐ Idade: _____
Pelagem: _____



Inspeção:
_____
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Índices paramétricos:

FC: _____ Pulso: _____ FR: _____ TR: _____ TPC: _____

Mucosas e linfonodos:
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_____

Sistema cardiovascular e respiratório:
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Sistema digestivo e urogenital:
_____
_____

Sistema locomotor:
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Sistema tegumentar e nervoso:
_____
_____

Observações:
_____
_____

Coleta de sangue: ☐

Eletrocardiograma: ☐

ANNEX III

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Prezado(a):

Esta é uma mensagem automática do sistema Solicite CEUA que indica mudança na situação de uma solicitação.

Protocolo CEUA**:** 388/2017
Título do projeto: Proteína recombinante contendo epítopos de células B de diferentes toxinas de aranha Loxosceles gera anticorpos neutralizantes em coelhos imunizados
Finalidade: Pesquisa
Pesquisador responsável: Carlos Delfin Chavez Olortegui
Unidade: Instituto de Ciencias Biologicas
Departamento: Departamento de Bioquimica e Imunologia

Situação atual: Decisão Final - Aprovado

Aprovado COM RECOMENDAÇÃO na reunião do dia 04/06/2018. Validade: 04/06/2018 à 03/06/2023 RECOMENDAÇÃO: Prezado pesquisador, o biotério onde sua pesquisa será realizada não está com cadastro e credenciamento finalizado no novo site do CIUCA/CONCEA. Solicitamos informar ao responsável pelo biotério para concluir o cadastro, porque em breve somente poderemos aprovar os que estiverem devidamente cadastrados e credenciados. Belo Horizonte, 05/06/2018.

Atenciosamente,

Sistema Solicite CEUA UFMG
https://aplicativos.ufmg.br/solicite_ceua/

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