PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
DOUTORADO EM FISIOPATOLOGIA E SAÚDE ANIMAL

PAULO FELIPE IZIQUE GOIOZO

ESTUDO COMPARATIVO DOS EFEITOS DO VENENO BRUTO DE SERPENTES *Crotalus durissus terrificus* (LAURENTI, 1768) E *Crotalus durissus collilineatus* (AMARAL, 1926) EM LESÕES TECIDUAIS OCASIONADAS PELA INTOXICAÇÃO EXPERIMENTAL EM RATOS WISTAR

Presidente Prudente - SP
2018

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Tese apresentada Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Doutor – Área de concentração: Fisiopatologia e Saúde Animal

Orientadora:
Profª Dra. Rosa Maria Barili Nogueira

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Presidente Prudente, 03 de Dezembro de 2018

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DEDICATÓRIA

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“Há três virtudes essenciais para o equilíbrio e sucesso na vida...

-Ensinar o que sabe...

-Praticar o que ensina...

-Questionar o que se ignora...

”. (Adaptado por Mário Sérgio Cortella do pensador britânico Beda – Século. VIII)

RESUMO

Estudo comparativo dos efeitos do veneno bruto de serpentes C*rotalus durissus terrificus* (Laurenti, 1768) e C*rotalus durissus collilineatus* (Amaral, 1926) em lesões teciduais ocasionadas pela intoxicação experimental em ratos wistar

Crotalus durissus é a única espécie do gênero que habita o Brasil e é responsável pelo maior número de mortes por acidentes ofídicos. O veneno crotálico é uma mistura complexa de proteínas ativas, enzimas, toxinas e polipeptídeos. Esses componentes possuem ações neurotóxicas, miotóxicas, nefrotóxicas, hepatotóxicas e coagulantes, no entanto, estudos mostram variações no efeito do veneno. O objetivo deste estudo foi quantificar, qualificar e diferenciar as lesões teciduais sistêmicas produzidas pelo veneno das serpentes *Crotalus durissus terrificus* e *Crotalus durissus collilineatus* em ratos Wistar. Utilizou-se ratos machos Wistar (n = 60). Os animais foram alocados em três grupos (n = 20 cada): Grupo Controle (GC); Grupo C. durissus terrificos (CdtG); e, grupo de serpentes C. durissus collilineatus (CdcG). Após a eutanásia, cérebro, pulmão, coração, fígado, jejuno, cólon, baço, rins, músculo esquelético e testículos foram coletados para análises histopatológicas e um escore de lesão por animal foi estabelecido. No sistema nervoso central, escores significativos das lesões ($p < 0,05$) foram observados no CdcG e no CdtG. O maior escore lesional foi observado no CdtG. No tecido muscular, o CdcG apresentou maior escore de lesão no coração, enquanto no músculo esquelético o maior escore foi encontrado no CdtG. No rim, lesões significativas foram observadas no CdcG e no CdtC com o escore lesional médio mais alto no CdcG. O maior escore de lesão hepática foi no CdtG. No pulmão, maior lesional elevado foi observado apenas no CdcG. No baço, baixos escores lesionais foram evidenciados. Nos intestinos, o cólon apresentou baixos escores de lesão, porém diferenças significativas ($p < 0,05$) foram observadas. O único órgão que apresentou diferença significativa ($p < 0,05$) entre os três grupos foi o jejuno. Nos testículos escores lesionais significativos foram encontrados em ambos os grupos. Nossos resultados mostram que o veneno crotálico tem um efeito deletério em todos os sistemas orgânicos. Ao abordar um paciente com suspeita de picada de cascavel, o clínico deve considerar variações nos sinais clínicos em virtude da composição, bem como diferenças no desempenho de cada subespécie em diferentes tecidos. Os dados obtidos em nosso estudo fornecem subsídios para novas pesquisas sobre a toxicidade do veneno crotálico das diferentes subespécies do gênero *Crotalus durissus.*

Palavras-chave: Escores Lesionais, Cascavéis e Histopatologia

ABSTRACT

Comparative study of the effects of the raw venom of snakes C*rotalus durissus terrificus* (Laurenti, 1768) and C*rotalus durissus collilineatus* (Amaral, 1926) on tissue injury caused by experimental intoxication in wistar rats

Crotalus durissus is the only species of the genus that inhabits Brazil and is responsible for the highest number of deaths from ophidian accidents. Crotalic venom is a complex mixture of active proteins, enzymes, toxins, and polypeptides. These components have neurotoxic, myotoxic, nephrotoxic, hepatotoxic and coagulant actions, however, studies show variations in the effects of the poison. The objective of this study was to quantify, characterize and differentiate the systemic tissue lesions produced by venom *of Crotalus durissus terrificus* and *Crotalus durissus collilineatus* snakes in Wistar rats. Male Wistar rats (n = 60) were used. The animals were allocated into three groups (n = 20 each): Control Group (CG); C. durissus terrificus group (CdtG); and C. durissus collilineatus group (CdcG). After euthanasia, brain, lung, heart, liver, jejunum, colon, spleen, kidneys, skeletal muscle and testes were collected for histopathological analysis and a lesion score per animal was established. In the central nervous system, significant lesion scores ($p < 0.05$) were observed in the CdcG and CdtG. The highest lesion score was observed in the CdtG. In muscle tissue, the CdcG presented a higher lesion score in the heart, whereas in the skeletal muscle the highest score was found in the CdtG. In the kidney, significant lesions were observed in the CdcG and CdtC with the highest mean lesion score in the CdcG. The highest hepatic injury score was in the CdtG. In the lungs, a higher lesion score was observed only in the CdcG. In the spleen, low lesion scores were evidenced. In the intestines, the colon presented low lesion scores, but significant differences ($p < 0.05$) were observed. The only organ that presented significant differences ($p < 0.05$) between the three groups was the jejunum. In the testis, significant lesion scores were found in both groups ($p<0,05$). Our results show that crotalic venom has a deleterious effect on all organic systems. When addressing a patient whit a suspected rattlesnake bit, the clinician should consider variations in clinical signs by virtue of the composition as well as differences in the performance of each subspecies in different tissues. The data obtained in our study provide subsidies for new research on the toxicity of crotalic venom of the different subspecies of the genus Crotalus durissus.

Keywords: Lesion Score, Rattlesnake and Histopathology.

SUMÁRIO

ARTIGO 1

COMPARATIVE STUDY OF TISSUE LESIONS CAUSED *BY CROTALUS DURISSUS TERRIFICUS* AND *CROTALUS DURISSUS COLLILINEATUS* VENOMS IN WISTAR RATS.[1]

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SUMMARY

Crotalus durissus snakes are responsible for the highest number of snakebite deaths in Brazil and countries in South and Central America. Crotalic venom is a complex mixture of active proteins, enzymes, toxins, and polypeptides. These components have neurotoxic, myotoxic, nephrotoxic, hepatotoxic and coagulant actions, however, studies show variations in the effects of the poison. The objective of this study was to quantify the systemic tissue lesions produced by venom of *Crotalus durissus terrificus* and *Crotalus durissus collilineatus* snakes in Wistar rats. Wistar males were divided into three groups (n = 20 each): Control Group (CG); *C. durissus terrificos* group (CdtG); and *C. durissus collilineatus* group (CdcG). After euthanasia, brain, lung, heart, liver, jejunum, colon, spleen, kidneys and skeletal muscle were collected for histopathological analysis and a lesion score per animal was established. Circulatory changes, inflammatory, degenerative and necrotic processes were found in the tissues analyzed. Cdt venom presented higher lesion scores in brain, skeletal muscle, liver and spleen. More severe lesions caused by Cdc venom were found in the heart, kidneys, lung, jejunum, and colon. Our results show that crotalic venom has a toxic effect on all organic systems. When addressing a patient with a suspected of rattlesnake bit, the clinician should consider variations in clinical signs by virtue of the composition as well as differences in the performance of each subspecies in different tissues. The data obtained in our study provide subsidies for new research on the toxicity of crotalic venom of the different subspecies of the genus *Crotalus durissus* as well as other vipers with similar venom composition.

KEY WORDS: Lesion Score, Rattlesnake and Histopathology.

INTRODUCTION

Crotalus durissus is responsible for the highest number of deaths due to ophidian accidents, constituting a problem both in public health and animal production, with more than 27.000 notifications registered in Brazil in 2014 [1-3]. This species is divided into seven subspecies of which *Crotalus durissus terrificus (Cdt)* and *Crotalus durissus collilineatus (Cdc)* stand out as they inhabit areas of greater population density in Brazil [4-6]. Crotalic venom is a complex mixture of active proteins, enzymes, toxins, and polypeptides. These components have neurotoxic, myotoxic, nephrotoxic, hepatotoxic and coagulant actions, however, studies show variations in the effect of venom according to subspecies, diet, habitat and the age of the snakes [3, 7-10].

The aim of this study was to quantify and qualify the systemic tissue lesions produced by the venom of the snakes *Crotalus durissus terrificus* and *Crotalus durissus collilineatus* in Wistar rats.

MATERIALS AND METHODS

Ethics Statements

This research was approved by the Ethics Committee and for Use of Animals (CEUA) of our institution in accordance with the National Council of Control and Animal Experimentation of Brazil (CONCEA), protocol nº 3220.

Animals

Sixty six-month-old male Wistar rats (350g) were used in the present study. The animals were housed in a ventilated environment in clean and sanitized cages with free access to food and water *ad libitum*, under a 12-h light: 12-h dark cycle and controlled temperature (22±2 Cº).

Venoms

Crotalus durissus terrificus and *Crotalus durissus collilineatus* lyophilized samples were kept frozen at -20ºC until the moment of use and reconstitution was performed at the concentration of 1.0mg / kg with saline solution 0.9%, according previous studies [12]. The venom management was in accordance with *WHO Guidelines for the Production Control and Regulation of Snake Antivenom Immunoglobulins* [13].

Experimental Procedures

The animals were submitted to the following treatments (20 rats/treatment): Control group (CG) inoculated with saline solution 0,9% intramuscularly; *C. durissus terrificus* group (CdtG) inoculated with 1,0mg/Kg of venom intramuscularly; and, *C. durissus collilineatus* group (CdcG) inoculated with 1,0mg/Kg of venom intramuscularly. The animals remained intoxicated for six hours according to previous clinical studies [14] and were subsequently submitted to inhalation anesthesia with isoflurane to induce unconsciousness verified by the absence of tail, interdigital and corneal reflexes and then euthanized by exsanguination [15].

Histological Assessment

After euthanasia, samples of brain, lung, heart, liver, jejunum, colon, spleen, kidneys, and skeletal muscle were collected and fixed in 10% buffered formalin solution, dehydrated in alcohols and embedded in paraffin. Sections of 3 µm were stained with Hematoxylin and Eosin (H&E), Masson trichrome and Periodic Acid-Schiff (PAS) for histopathological evaluation.

Histopathological analyzes were performed throughout the histological section. A histological score adapted from previous studies [16-18] was used to compare morphological changes and lesions between the treatments (Table 1).

93 **Table – 1:** Lesion score-endpoint* used to evaluate histologic lesions in rats
94 experimentally inoculated with *Crotalus durissus terrificus* or *Crotalus durissus*
95 *collilineatus* venom
96

Tissue	Type of Lesion	Severity Factor	Maximal Score
Brain	Red Neuron	3	45
	Gliosis	2	
	Central Chromatolysis	3	
	Malacia	3	
	Wallerian degeneration	2	
	Congestion	2	
Lung	Alveolar edema	2	21
	Congestion	2	
	Interstitial inflammatory infiltrate	2	
	Hemorrhage	1	
Heart	Congestion	2	45
	Inflammatory infiltrate	2	
	Interstitial edema	2	
	Hemorrhage	2	
	Myocyte degeneration	2	
	Myocyte hypertrophy	2	
	Necrosis	3	
Liver	Apoptosis	2	36
	Cytoplasmic vacuolation	1	
	Disorganization of hepatocytes cords	1	
	Inflammatory infiltrate	2	
	Megalocytosis	2	
	Nuclear vacuolations	1	
	Necrosis	3	
Jejunum	Cubic enterocyte	1	39
	Enterocyte vacuolation	2	
	Interstitial edema	2	
	Lymphatic vessel dilatation	1	
	Villi apical necrosis	3	
	Villus fusion	2	
	Villus flattening	2	
Colon	Cubic enterocyte	2	24
	Interstitial edema	2	
	Lymphatic vessel dilatation	1	
	Necrosis	3	
Spleen	Apoptosis	1	36
	Germinal center depletion	1	
	Germinal center proliferation	1	
	Histiocytosis	2	
	Inflammatory Infiltrate	2	
	Mitosis	1	
	Necrosis	3	
	Reactivity	1	
Kidneys	Congestion	1	33
	Cytoplasmic vacuolation	1	
	Inflammatory infiltrate	2	
	Hemorrhage	2	
	Necrosis	3	
	Nuclear vacuolation	1	
	Tubular casts	1	
Skeletal muscle	Congestion	2	45
	Inflammatory infiltrate	2	
	Interstitial edema	2	
	Hemorrhage	2	
	Myocyte degeneration	2	
	Myocyte hypertrophy	2	
	Necrosis	3	

*The score for each lesion was obtained by multiplying the severity factor by the extent of the lesion. The organ score was then obtained through the sum of each lesion score. Severity factor (degree of severity): 1-mild lesions, 2-moderate lesions and 3-severe lesions. The extent of each lesion (intensity or observed frequency) was evaluated and scored as 0-no lesion, 1-low extent (25% of the tissue affected), 2-intermediate extent (50% of the tissue affected) and 3- large extent (75% or more of the tissue affected).

Statistical Analysis.

The normality and homogeneity of data ware previously analyzed using the Shapiro-Wilk and Bartlett tests respectively. When these two assumptions were met, the analysis of variance (ANOVA) with Duncan's test contrast was used to determinate statistical differences between groups Statistical significance was set at *P>0.05* [19].

RESULTS

Six hours after venoms exposure, the rats presented discrete to severe histological lesion compared with the control (Table 2).

Table – 2: Lesion scores obtained in different tissues of Wistar rats inoculated with *Crotalus durissus collilineatus* or *Crotalus durissus terrificus* venom.

Tissue	Control	CdcG	Cdt
Brain	3.04±1.62[a]	9.63±6.01[b]	14.10±0.85[b]
Skeletal muscle	0.70±0.97[a]	12.0±4.51[b]	14.88±2.2[b]
Heart	6.90±1.65[a]	13.58±3.74[b]	11.75±3.40[b]
Kidney	2.42±0.60[a]	13.78±3.58[b]	11.50±2.46[b]
Liver	3.68±0.67[a]	10.63±1.46[b]	13.72±2.96[b]
Lung	4.20±1.70[a]	9.05±2.14[b]	3.60±2.01[a]
Spleen	1.12±0.34[a]	3.35±0.93[b]	3.47±1.12[b]
Jejunum	0.10±0.30[a]	10.47±2.96[b]	7.47±2.91[c]
Colon	0.85±0.93[a]	4.88±2.11[b]	4.64±2.34[b]

Different letters on the same line (a,b and c) denote significant differences (p<0.05); ***CdcG:*** Crotalus durissus collilineatus group; ***CdtG:*** Crotalus durissus terrificus group.

In the central nervous system, significant lesion scores (p <0.05) were observed in the CdcG and CdtG groups in relation to the control group. The lesions presented a discrete distribution characterized by Wallerian degeneration, red neurons, central chromatolysis, gliosis and congestion (Figure 1A).

In the muscle tissue (heart and skeletal muscle), the CdcG group presented a higher lesional score-endpoint in the heart whereas in the skeletal muscle tissue the highest score was found in the CdtG group. In the CdcG and CdtG groups the lesions in the heart observed were inflammatory infiltrate with mononuclear predominance, interstitial edema, hemorrhagic foci, degeneration and myocyte necrosis with discrete distribution (Figures 1B and 1C).

In the renal parenchyma, the most frequent lesions were degeneration and tubular necrosis followed by circulatory changes (vascular congestion and hemorrhage) and nuclear vacuolization (Figure 1D).

In the liver, the main histological lesions were cytoplasmic vacuolization, hepatocyte cord disruption, nuclear vacuolization and coagulation necrosis (Figure 1E). In the lungs, an elevated lesional score-endpoint was observed only in the Cdc group. The changes with moderate distribution in the CdcG constituted circulatory changes such as vascular congestion and hemorrhage (Figure 1F).

Low lesion score-endpoints were observed in the spleen, however, significant differences ($p < 0.05$) were observed in the CdcG and CdtG groups in relation to the control group. Only germinal center depletion was evidenced with discrete intensity (Figure 1G).

In the intestines, the colon presented low lesion scores, however, significant differences ($p < 0.05$) were observed between the CdcG and CdtG when compared to the CG. In this organ, only necrosis and dilatation of lymphatic vessels with discrete distribution were observed in both inoculated groups (Figure 1H). In this experiment, the only organ that presented significant differences ($p < 0.05$) between the three groups was the jejunum. The CdcG group presented a higher lesional score-endpoint with lesions in the villi (apical necrosis, flattening and fusion of villi), dilatation of

lymphatic vessels and cubic enterocytes. In the CdtG group, only villous changes and dilation of lymphatic vessels were observed (Figure 1I).

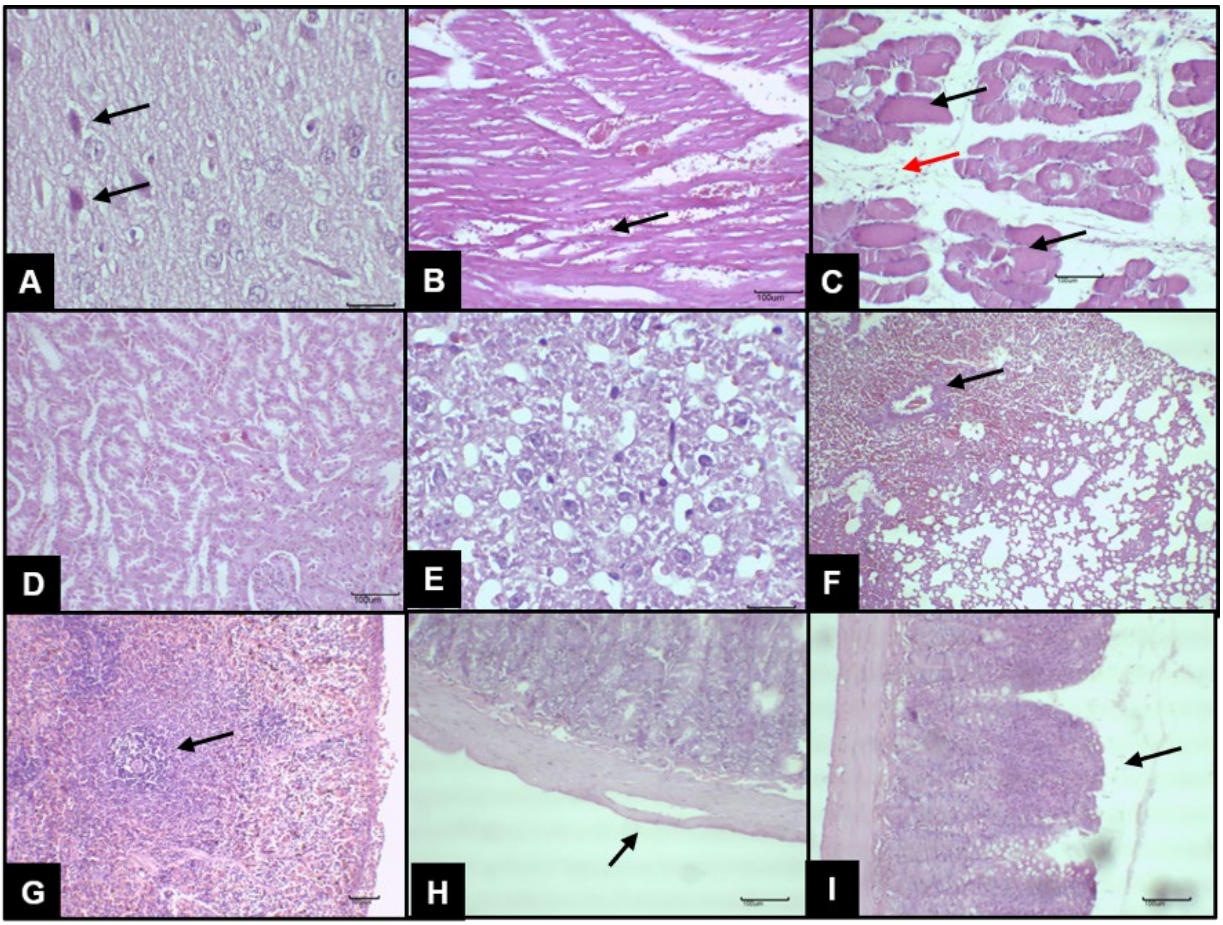

Figure 1: Photomicrographs of different tissues of Wistar rats inoculated with snake venom Crotalus durissus sp. (H&E, Bar 100 µm) (**A)** Brain (CdtG) showing red neurons (black arrow); **(B)** Heart (CdcG). Hemorrhagic areas (black arrow); **(C)** Skeletal muscle (CdtG). Interstitial mononuclear inflammatory infiltrate (black arrow) and fibers with coagulation necrosis (red arrow); **(D)** Kidney (CdcG). Areas with hemorrhage and tubular cells with pyknotic nuclei and karyolysis; **(E)** Liver (CdtG) showing marked cytoplasmic vacuolization and pyknotic nuclei; **(F)** Lung (CdcG) with marked circulatory changes (black arrow); **(G)** Spleen (CdtG) with discrete lymphoid germinal center depletion (black arrow); **(H)** Large intestine (CdcG) showing dilatation of lymphatic vessel (black arrow); **(I)** Small intestine (CdcG) showing fusion of the villi (black arrow).
CdcG = Crotalus durissus collilineatus group; ***CdtG*** = Crotalus durissus terrificus Group.

DISCUSSION

Studies involving the toxic effects of venom have been performed in isolated organs such as kidney and liver [7,9,20,21], or only in fractions such as crotamine, crotoxin or LAAO (L-amino acid oxidase) [8,10,22-25].

Crotamin is a major component of crotalic venom and has demonstrated toxicity and proinflammatory activity nervous and systemic [24]. In addition, preliminary studies indicate that thrombin-like enzyme isolated from *Crotalus durissus terrificus* venom induces in vivo neurotoxicity resulting morphological and behavioral in brain tissue [26]. These effects are consistent with the tissue lesions found. The *C.d. terrificus* venom resulted in late-to-endpoint scores above those of *C. d. collilineatus*. This corresponds to the low peripheral toxicity caused by the action of the crotamine-negative fraction present in the venom of *C. d. collilineatus* where this fraction is responsible for analgesia in cases of accidents with this subspecies [21]. The muscular lesions in crotalic accidents are caused by the association of crotoxin-simile and phospholipase A2 (PLA2), being present in the venom of all subspecies [6,8]. Crotamine also acts on muscle degradation by presenting the capacity of cellular penetration and modifying cellular functions, mainly from mitochondria [28,29]. In our study, we demonstrated muscle lesions with higher lesion scores in animals inoculated with the venom of *C. d. collilineatus*, although elevated lesion scores were also shown in animals inoculated with venom of *C. d. terrificus*.

Controversial findings of the inoculated venoms were observed in the heart, in which, greater lesions were seen in the animals inoculated with venom of *C. d. collilineatus*. These results indicate that cardiac lesions could be associated with lung lesions, since only the animals of the CdcG group demonstrated alterations in the lungs. Pulmonary changes in ophidian accidents are poorly described in the literature,

192 studies with the subspecies *C.d. cascavela* have shown that the action of crotamine
193 with PLA2 can induce circulatory changes in the lungs [29]. Our results reveal the
194 differences between the subspecies studied suggesting further studies are necessary
195 on the respiratory dynamics in the crotalic poisoning of different subspecies.
196 In the liver, hepatotoxicity from the venom of *C. d. terrificus* occurs as a consequence
197 of vascular lesions resulting in increased serum hepatic enzymes and structural
198 damage [9]. The results obtained in the present study indicate that the difference in
199 venom composition may have direct effects on hepatocytes, since the CdtG
200 presented a higher lesional score-endpoint can due the greater intensity in the
201 injured hepatocytes. Our results associated with previous studies may suggest that
202 these morphological changes are due to the actions of crotamine in mitochondria,
203 which depending on the concentration and time of exposure may induce apoptosis or
204 result in cell death through oxidative stress [22,28,30].
205 The majority of deaths due to crotalic accidents are attributed to acute renal failure
206 caused by venom. Studies indicate that acute renal failure results from the effects of
207 crotamine on the glomeruli together with changes in renal hemodynamics and
208 vascular changes with decreased blood flow [20,21]. In both inoculated groups, high
209 lesion scores were observed, being more pronounced in the animals inoculated with
210 *Crotalus durissus collilineatus* venom. Studies have shown that *Crotalus durissus*
211 *collilineatus* venom induces direct and indirect morphofunctional changes in the
212 kidneys due to the synergism of crotoxin and PLA2 and the release of other
213 substances such as myoglobin [31]. As shown in our results, there are significant
214 differences between the subspecies, indicating that the composition of the venom
215 directly influences the renal damages.

As described previously, crotalic venom has a broad performance in different organ systems due to its complex composition, which results in nonspecific clinical signs that make diagnosis difficult [3]. This study reports splenic and intestinal morphological changes caused by crotalic poisoning by the subspecies *C. d. terrificus* and *C. d. collilineatus* previously not described in the literature, noting significant differences between subspecies were detected in the small intestine. In the spleen, we may suggest that the changes are due to the suppressive action of crotamine demonstrated by previous studies [32].

CONCLUSION

In conclusion, our results show that crotalic venom has a deleterious effect on all organ systems. When approaching a patient with a suspected rattlesnake bite, the medical clinician should consider variations in clinical signs by virtue of composition as well as differences in the performance of each subspecies in different tissues. The data obtained in our study provide subsidies for new research on the toxicity of crotalic venom of the different subspecies of the genus *Crotalus durissus.*

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ARTIGO 2

Acute testicular toxicity *of Crotalus durissus terrificus* and *Crotalus durissus collilineatus* venom in Wistar rats: A histological comparative study

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Acute testicular toxicity *of Crotalus durissus terrificus* and *Crotalus durissus collilineatus* venom in Wistar rats: A histological comparative study

ABSTRACT:

The species *Crotalus durissus* is responsible for the highest number of deaths due to ophidian accidents. Although widely researched, there are no studies on the effects of crotalic venom on the tissue morphology of the male reproductive system. *Crotalus durissus terrificus* and *Crotalus durissus collilineatus* crotalic venom (1.0 mg / kg) resulted in significant tissue damage ($p < 0.05$), mainly cytoplasmic vacuolization, basement membrane changes and spermatogonia necrosis. In conclusion, our results show that spermatogenesis is compromised by tissue damage caused by crotalic poisoning.

Keywords: Rattlesnakes; lesional score, spermatogenesis.

The species *Crotalus durissus* is the only one of the genus in South America, being found from Mexico to the North of Argentina. In Brazil, seven sub-species are found; *Crotalus durissus terrificus, Crotalus durissus collilineatus, Crotalus durissus cascade, Crotalus durissus ruruima, Crotalus durissus marajoensis, Crotalus durissus dryinus* and *Crotalus durissus trigonicus* (Uetz, 2006), which inhabit dry, sandy, rocky and rarely coastal areas (Houos and Almeida-Santos, 2016). The venom is a complex mixture of biologically active proteins, enzymes, toxins, and polypeptides that exhibits variation in toxic activity and composition both among subspecies and among individuals of the same subspecies due to variations in diet, habitat and age (Boldrini-França et al., 2010; Dos Santos, 2014; França et al., 2009; Lourenço et al., 2013; Oliveira et al., 2018). Studies show that crotalic venom has neurotoxic, coagulant, myotoxic, nephrotoxic, and hepatotoxic actions (Albuquerque

et al., 2013; Fernandes, 2008; França et al., 2009), in animals and humans, however, the deleterious effects on the tissue morphology of the gonads are unknown. Additionally, epidemiological data and governmental statistics indicate that crotalic accidents are responsible for the highest number of deaths in humans and animals (Bastos et al., 2005; Brasil, 2015; Estrada et al., 2014). Due to the complexity and variation of the composition of the crotalic venom, the objective of the present study was to evaluate the effents on tissue in the testes of rats, comparing two subspecies of *Crotalus durissus*.

Sixty male rats ninety days old, of the Wistar lineage were used. The animals were kept in in an environment with a controlled temperature (22±2 Cº) and light / dark cycle of 12 hours. All protocols and behaviors involving the animals of the present study were approved by the Ethics Committee on the Use of Animals under protocol number 3220 (UNOESTE-Brazil).

Lyophilized samples of venoms of *Crotalus durissus terrificus* and *Crotalus durissus collilineatus* were kept frozen at -20º C until the moment of use.

The animals were randomly assigned to three treatments (n = 20): Control Group (CG) inoculated with 0.9% saline solution intramuscularly; *Crotalus durissus terrificus* (Cdt) inoculated with 1.0 mg / kg of venom intramuscularly and *Crotalus durissus collilineatus* (Cdc) inoculated with 1.0 mg / kg of venom (Motta et al. 2009). Six hours after inoculation, according clinical previous studies (Mise et al. 2018), the animals were euthanized by inhalation of anesthesia with isoflurane and subsequent exsanguination.

The testes were collected and fixed in 10% buffered formalin solution for 48 hours. The tissue samples were embedded in paraffin and the blocks were cut in a semi-

automated microtome than 3 µm sections were stained with Hematoxylin and Eosin (HE) and Periodic Acid Schff (PAS).

For histopathological evaluation, a testicular histological score was used. (Gibson-corley et al., 2013; Mann et al., 2012). The testicular lesion score was obtained by multiplying the severity factor by the extent of the lesion. The organ score was then obtained by the sum of each lesion score. The severity factor (degree of severity) was assessed as: 1-mild lesions, 2-moderate lesions and 3-severe lesions. The extent of each lesion (intensity or observed frequency) was evaluated and scored as 0-no lesion, 1-low extent (25% of the tissue affected), 2-intermediate extent (50% of the tissue affected) and 3- large extent (75% or more of the tissue affected). Vascular congestion (Severity factor = 1), interstitial edema (Severity factor = 1), basal membrane integrity (Severity factor = 2), sperm cells cytoplasmic vacuolization (Severity factor = 2) and sperm cells necrosis (Severity factor = 3) were considered to determine the maximum lesional score.

The data obtained were evaluated for normality and homogeneity by the Shapiro-Wilk and Bartlett tests, respectively. Subsequently, a non-parametric analysis of variance (ANOVA) was performed using the Kruskal-Wallis test with Dunn's test to determine the differences between the groups, with a significance level of 5% ($p < 0.05$) (Dinno, 2017). All analyzes were performed with Software R Studio (Version 3.4.0) (R Core Team, 2017).

The testes (right and left) of the control, Cdc, and Cdt rats were collected and submitted to histopathological evaluation. Animals of the control group presented only mild peripheral interstitial edema and mild vascular congestion. Higher lesional score were observed in the Cdt, and Cdc when compared with the control group (Fig. 1).

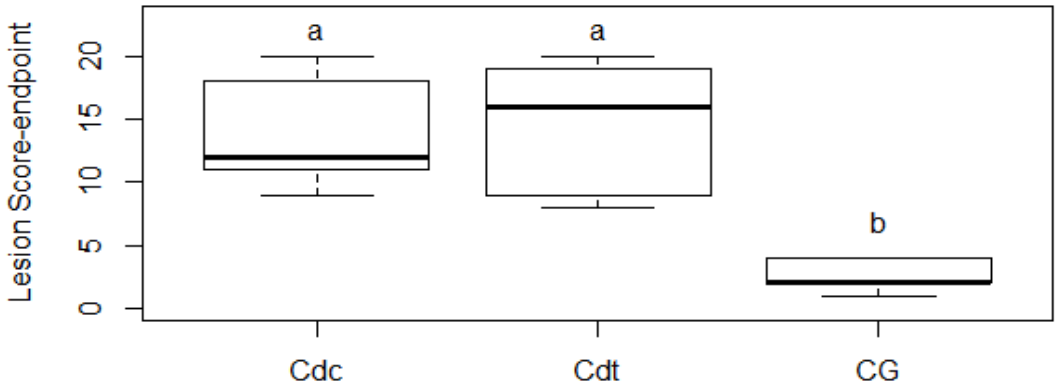

Figure – 1: Median of the lesion score-endpoint of the testis of rats inoculated with crotalic venom. The line in the center denotes the median, the upper and lower edges indicate the interquartile deviation and the outside lines indicate the maximum and minimum values. Equal letters (a) do not differ from each other (P> 0.05).

In groups of poisoned animals, cytoplasmic vacuolization was observed in more than 75% of the tubules. The integrity of the basement membrane (Fig. 2) was affected in approximately 50% of the tubules, as well as the frequency of spermatogonia necrosis. Peripheral interstitial edema and discrete vascular congestion were also observed in the Cdt and Cdc.

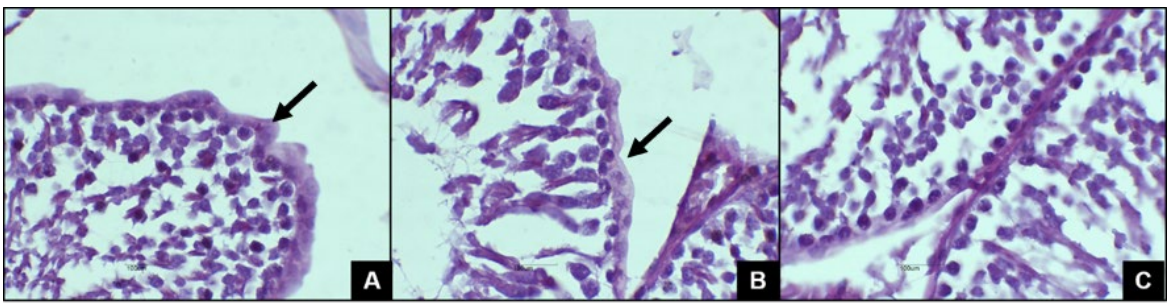

Figure – 2: A: Seminiferous tubules, in **A** (Cdc) and **B** (Cdt) It is observed a basal membrane with irregular contour and weakly stained (arrow) (PAS, 400x). **C** (CG)

Seminiferous tubules with preserved and strongly stained basement membrane (PAS, 400x).

Our results confirm those of a previous study (Fernandes et al., 2018) that demonstrated that the venom of *Crotalus durissus sp*. affects spermatogenesis resulting in increased morphological abnormalities and decreased sperm concentration.

The effects of crotalic venom have been widely studied in recent decades and significant advances have been reported in both toxic tissue action (Dos-santos, 2014) and the pharmaceutical use of substances that make up the venom such as crotamine (Batista da Cunha et al., 2018). The present study, in spite of all the investigations involving the actions and uses of rattlesnake venom of the species *Crotalus durissus*, is the first to describe the tissue damages caused in the testicles.

Tissue lesions in the testicles by toxic agents may act by breaking the blood-testicular barrier, inducing exfoliation of germ cells (Gao et al., 2016) or degenerative and necrotic processes of spermatogonia (Tabassum et al., 2017). Among the components of crotalic venom, crotoxin has edematogenic, inflammatory and necrotizing action and phospholipase (PLA2) presents important cytotoxic potential (Silva et al., 2012). In our study, the control group (CG) exhibited normal spermatogenesis, with an intact basement membrane and the presence of all cells of the spermatogenic lineage, as well as spermatozoids in the tubular lumen. In the poisoned groups (Gdc) and (Gct), degenerative and necrotic processes were observed at the same intensity ($p > 0.05$), indicating the effects of the crude venom on the testicular tissue.

In the testicles, toxic agents penetrate the seminiferous tubules through a selective influx from the apical complex, acting rapidly on the cytoskeletal actin (Su et al.,

2011). As observed in our results, the dose used and short time period (1mg/Kg for six hours) demonstrated that crotalic venom demostrates the properties of breaking the hematopoietic barrier and affecting spermatogenesis.

The mechanisms involved in the degenerative and necrotic processes of spermatogonia may be associated with the penetrating capacity of crotamine, leading to damage to mitochondrial membranes (Batista da Cunha et al., 2018). In addition, exposure time and venom concentration are directly related to clinical severity (Mise et al., 2018), and may induce apoptosis or necrosis through the release of cytochrome C, oxidative stress and disruption of ATP synthesis (da Silva et al., 2018; Yan et al., 2006).

Ischemic injuries result in severe testicular lesions leading to the detachment of Sertoli cells from the basal membrane and degeneration of spermatogonia (Yazama et al., 2015). In addition, crotamine is a fraction of the crotalic venom that promotes platelet aggregation (Batista da Cunha et al., 2018), and in this context, vascular events should be considered as adjuvants in testicular injury caused by crotalic poisoning, since this polypeptide is present in the venom of *Crotalus durissus terrificus* and *Crotalus durissus collilineatus* (Oliveira et al., 2018).

This study concludes that exposure to venom of the subspecies *Crotalus durissus terrificus* and *Crotalus durissus collilineatus* induced acute testicular toxicity and reduced spermatogenesis, indicating the need to monitor the fertility of humans and animals.

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ANEXO 1 - PARECER FINAL DA COMISSÃO DE ÉTICA E USO DE ANIMAIS (CEUA/UNOESTE)

UNOESTE - Universidade do Oeste Paulista

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO

PPG - Programa de Pesquisa de Pós-Graduação
PEIC - Programa Especial de Iniciação Científica

Parecer Final

Declaramos para os devidos fins que o Projeto de Pesquisa intitulado **"ESTUDO COMPARATIVO DOS EFEITOS DO VENENO BRUTO DE SERPENTES CROTALUS DURISSUS TERRIFICUS (LAURENTI, 1768) E CROTALUS DURISSUS COLLILINEATUS (AMARAL, 1926) EM LESÕES SISTÊMICAS OCASIONADAS PELA INTOXICAÇÃO EXPERIMENTAL EM RATOS WISTAR"**, cadastrado na Coordenadoria de Pesquisa, Desenvolvimento e Inovação (CPDI) sob o número nº **3220** e tendo como participante(s) **ROSA MARIA BARILLI NOGUEIRA (responsável), KARINE MARIELE GUIMARAES DE CASTRO (discente), LILIANE GIROTO PEREIRA (discente), PAULO FELIPE IZIQUE GOIOZO (discente)**, foi avaliado e **APROVADO** pelo **COMITÊ ASSESSOR DE PESQUISA INSTITUCIONAL (CAPI) e COMISSÃO DE ÉTICA USO DE ANIMAIS (CEUA)** da Universidade do Oeste Paulista - UNOESTE de Presidente Prudente/SP.

Este Projeto de Pesquisa, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de Outubro de 2008, do Decreto nº 6.899, de 15 de Julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), tendo sido APROVADO em reunião realizada em 08/06/2016.

Vigência do projeto: 05/2016 a 09/2018.

Espécie/Linhagem	Nº de Animais	Peso	Idade	Sexo	Origem
Ratos Wistar	84	350 gramas	180 dias	M	Biotério

Presidente Prudente, 17 de Junho de 2016.

Prof. Dr. Jair Rodrigues Garcia Jr.
Coordenador Científico da CPDI

Profª Ms. Adriana Falco de Brito
Coordenadora da CEUA - UNOESTE

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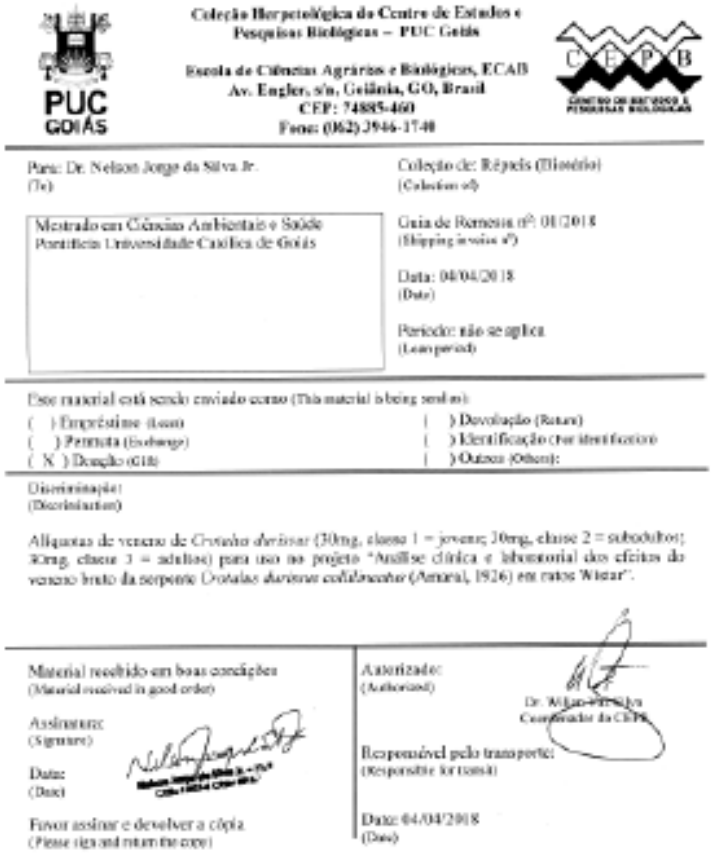
ANEXO 2 - TERMO DE DOAÇÃO DOS VENENOS UTILIZADOS NO ESTUDO



Coleção Herpetológica do Centro de Estudos e Pesquisas Biológicas – PUC Goiás

Escola de Ciências Agrárias e Biológicas, ECAB
Av. Engler, s/n, Goiânia, GO, Brasil
CEP: 74885-460
Fone: (062) 3946-1748

Para: Dr. Nelson Jorge da Silva Jr.
(To)

Mestrado em Ciências Ambientais e Saúde
Pontifícia Universidade Católica de Goiás

Coleção de: Répteis (Herpetologia)
(Collection of)

Guia de Remessa nº: 01/2018
(Shipping invoice nº)

Data: 04/04/2018
(Date)

Período: não se aplica
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( ) Permuta (Exchange)
(X) Doação (Gift)
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( ) Outros (Others):

Discriminação:
(Description)

Alíquotas de veneno de *Crotalus durissus* (30mg, classe 1 = jovens; 30mg, classe 2 = subadultos; 30mg, classe 3 = adultos) para uso no projeto "Análise clínica e laboratorial dos efeitos do veneno bruto da serpente *Crotalus durissus collilineatus* (Amaral, 1926) em ratos Wistar".

Material recebido em boas condições
(Material received in good order)

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Acta Veterinaria

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Type the manuscript double-spaced, using 12 font size and 3,0 cm margins. The journal requires line numbering in submitted manuscripts. Number the pages consecutively with the title page being page 1. Original research articles, case reports and methodology articles should usually occupy no more than eight printed pages. Text headings should be typed in capitals and subheadings in *italics*with an only initial capital letter. The text may contain a few short subheadings.

Original research articles should report on original primary research. Original research articles should be subdivided in: Title page, Summary and Keywords, Introduction, Material and Methods, Results, Discussion, Acknowledgements, References, Tables / Figures / Illustrations (with Legends).

Methodology articles should present a new method, test or procedure. The method described may either be completely new, or may offer an improved version of an existing method. The article must describe a clear advantage over what is currently available. The method needs to have been well tested and ideally, but not necessarily, used in a way that proves its value. Methodology articles should be subdivided into Title page, Summary and Key words, Introduction, Methods - Results and Discussion, Conclusions, Acknowledgements, References, Tables, Figures, Illustrations (with Legends). The ***Accession Numbers*** of any nucleic acid sequences, protein sequences or atomic coordinates cited in the manuscript should be provided, in square brackets and include the corresponding database name.

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In Short communication, Results and Discussion section is the interpretation of the results and their relation to the existing knowledge. The contribution to Veterinary medicine must be clearly stated.

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The Title page should include:

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Below the end of English and Serbian Summary four to six Key words in alphabetical order should be provided, using the entries from Index Medicus for indexing purposes.

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The essence of the problem and the purpose of the study should be pointed at the introduction. References discussed in the manuscript should be cited.

Materials and Methods

Identify the methods and procedures in sufficient details to allow other workers to reproduce the results. If methods are widely known, they should not be described, but only references indicated. Give references to established methods including statistical methods. Specify any general computer program used. Identify all drugs and chemicals used with generic names, doses and route of administration. Provide manufacturer and product number where applicable.

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The following (or similar) statement should be included in the end of Material and methods section: *Informed consent: Informed consent has been obtained for client-owned animals included in this study.*

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Experimental research on vertebrates or any regulated invertebrates must comply with institutional (e.g. Institutional Animal Care and Use Guidelines), national (e.g. Law for animal welfare protection) and international guidelines (e.g. Directive 2010/63/EU in Europe). It is the responsibility of the authors to obtain approval of appropriate regulatory group and report this approval in their manuscript in the first paragraph of the section Materials and methods including the name of the regulatory group, reference number and date of the approval.

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Results

Results should be presented in logical sequence, using tables and figures without duplication. The data should be precise and expressed according to the International System of Units **(SI).**

Discussion

Results should be discussed and related to other relevant studies. The Conclusions should be linked with the goals of the study, avoiding unqualified statements and conclusions not supported by your data. The new information should be distinguished from the previous finding and relevant hypothesis may be generated.

Acknowledgements

Acknowledgements should be placed at the end of the text indicating financial support or technical assistans. Name of the funding organizations should be written in full.

References

Citations such as *personal communications*, *unpublished data* or *in press* are not accepted. Meeting abstract may be cited only if published in indexed journals. Only essential references should be included in the text by Arabic numerals in square brackets and numbered consecutively in the order in which they are mentioned in the text. Always the same number is indicated in square brackets for the reference which is cited several times in the text. Automatic numbering should be avoided. References are typed on sheets separate from the text and follow the text.

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Articles

Simpson VR, Davison NJ, Kearns AM, Pichon B, Hudson LO, Koylass M, Blackett T, Butler H, Rasigade JP, Whatmore AM: Association of a lukM-positive clone of *Staphylococcus aureus* with fatal exudative dermatitis in red squirrels (*Sciurus vulgaris*). Vet Microbiol 2013, 162:987–991.

Abstracts

Marinkovic D, Aleksic-Kovacevic S, Knezevic M: Verminous arteritis of the cranial mesenteric artery of horses: the role of arterial smooth muscle cells [abstract]. J Comp Pathol 2010, 143:351.

Book and Monographs

Munson L, Terio KA, Ryser-Degiorgis MP, Lane EP, Courchamp F: Wild felid diseases: conservation implications and management strategies. In: *Biology and conservation of wild felids.* Oxford, United Kingdom: Oxford University Press; 2010, 237-259.

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Journal of Comparative Pathology

Guide for Authors

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The *Journal of Comparative Pathology* exists to publish articles recording research and original scientific findings relevant to the diseases of domesticated and other vertebrate animals. Articles on diseases of man are also appropriate if they present features of special interest when viewed against the general background of vertebrate pathology.

In addition, the Journal may publish Short Papers. These are intended to include reports of small completed investigations, new techniques or case descriptions. They should not have the subdivisions of a full length paper, but should include a brief summary and essential references. They would normally not exceed a word limit of 2000 and should include no more than four supportive figures (as individual images not composites of multiple images) or tables. Such submissions should be clearly marked 'Short Paper'. Single case reports will be accepted only if they make a significant contribution to knowledge.

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