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Serum and immunohistochemical analyses of troponin I in sheep experimentally poisoned with *Palicourea marcgravii*[1]

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ABSTRACT.- Cunha I.M., Lessa D.A.B., Carvalho V.A.N., Santos B.B.N., Souza G.N., Alencar N.X., Russano K., Paes J.M., Chenard M.G. & Helayel M.J.S.A. 2024. **Serum and immunohistochemical analyses of troponin I in sheep experimentally poisoned with *Palicourea marcgravii***. *Pesquisa Veterinária Brasileira 44:e07422, 2024*. Faculdade de Veterinária, Universidade Federal Fluminense, Av. Almirante Ary Parreiras 503, Santa Rosa, Niterói, RJ 24230-340, Brazil. E-mail: isabellemedvet@gmail.com

Palicourea marcgravii is a lethal toxic plant widely distributed in Brazil. Ingestion of this plant causes cardiotoxic effects in animals, leading to acute heart failure without evident macroscopic changes in the heart due to the rapid progression of the disease. Currently, the diagnosis is confirmed based on microscopic identification of characteristic renal lesions. Although troponin is used as a biomarker for myocardial lesions in human and veterinary medicine, its serum levels in sheep poisoned with *P. marcgravii* remain unknown. The objective of this study was to determine serum levels of troponin I and evaluate its expression in incipient heart lesions in sheep. Eight male sheep were experimentally intoxicated with 1g kg^{-1} of fresh *P. marcgravii* plants. The animals were physically examined every two hours, and blood samples were collected before the administration of the plant material and during the agonizing period immediately before death. Additionally, necropsy and immunohistochemistry were performed on cardiac tissue samples. All animals presented minimal serum levels of troponin I before plant administration, with a significant increase in these levels immediately before death, indicating leakage due to the cardiac injury. These results confirm the hypothesis that troponin is released into the bloodstream before morphological changes in the myocardium can be observed through conventional microscopy and immunohistochemical testing. These findings contribute to understanding the pathological mechanisms of this toxicity and may assist in the early diagnosis and treatment of affected animals.

INDEX TERMS: Clinical pathology, immunohistochemical, ruminants, sheep, troponin I, toxic plants, *Palicourea marcgravii.*

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RESUMO.- [**Análises séricas e imuno-histoquímicas da troponina I em ovinos intoxicados experimentalmente com *Palicourea marcgravii*.**] *Palicourea marcgravii* é uma planta tóxica letal e amplamente disseminada no Brasil. Sua ingestão causa efeitos cardiotóxicos em animais, levando à insuficiência cardíaca aguda sem alterações macroscópicas evidentes no coração devido à rápida progressão da doença. A confirmação do diagnóstico atual se baseia na identificação microscópica das lesões renais características. Embora a troponina seja usada como biomarcador de lesões miocárdicas na medicina humana e veterinária, seus níveis séricos em ovinos intoxicados por *P. marcgravii* ainda são desconhecidos. O objetivo deste estudo foi determinar os níveis séricos de troponina I e avaliar sua expressão em lesões incipientes no coração de ovinos. Oito ovinos machos foram experimentalmente intoxicados com 1g/kg da planta fresca. Os animais foram avaliados fisicamente a cada duas horas, e tiveram seu sangue coletado antes da administração da planta e durante a fase agônica, antes do óbito. Além disso, foram realizadas necropsia e imunohistoquímica em fragmentos cardíacos. Todos os animais apresentaram níveis mínimos de troponina antes da administração da planta, e mostraram aumento expressivo imediatamente antes do óbito, indicando extravasamento devido à lesão cardíaca. Esses resultados confirmam a hipótese de que a troponina é liberada na corrente sanguínea antes das alterações morfológicas no miocárdio serem observadas pela microscopia convencional e imunohistoquímica. Esses achados contribuem para a compreensão dos mecanismos patológicos dessa intoxicação e podem auxiliar no diagnóstico precoce e tratamento em animais afetados.

TERMOS DE INDEXAÇÃO: Patologia clínica, imunohistoquímica, ruminantes, ovinos, troponina I, plantas tóxicas, *Palicourea marcgravii.*

INTRODUCTION

Palicourea marcgravii (Rubiaceae) is one of the most important toxic plants in Brazil (Nascimento et al. 2018). Found throughout Brazil, it inhabits regions with good rainfall, never occurring in floodplains. It grows well in semi-shades on the edge of forests. However, its survival is ephemeral in clean pastures exposed to the sun (Tokarnia et al. 2012). It contains sodium monofluoroacetate (MF) as the toxic compound (Oliveira 1963, Krebs et al. 1994, Moraes-Moreau et al. 1995, Nogueira et al. 2010, Peixoto et al. 2012), which causes cardiotoxic effects in cattle (Maxie & Robinson 2007, Nogueira et al. 2010), sheep (Schultz et al. 1982, Peixoto et al. 2010, Koether et al. 2019), horses, goats, rabbits, and monkeys, as well as neurotoxic effects in dogs, laboratory animals, rats, and hamsters (Chenoweth & Gilman 1946). Under natural conditions, cattle, sheep, goats, and possibly horses are affected mainly (Tokarnia et al. 2012). Experimentally, there are several studies on cattle (Pacheco & Carneiro 1932, Döbereiner & Tokarnia 1959, Camargo 1962, Costa et al. 1984, Tokarnia & Döbereiner 1986, Barbosa et al. 2003, Rodrigues 2015, Serodio et al. 2019), sheep (Tokarnia et al. 1986, Soto-Blanco et al. 2004, Barbosa 2016), goats (Tokarnia et al. 1991, Soto-Blanco et al. 2004, Barbosa et al. 2015), horses (Tokarnia et al. 1993), buffaloes (Barbosa et al. 2003), rabbits (Peixoto et al. 1987), rats (Peixoto et al. 2011) and guinea pigs (Pacheco & Carneiro 1932, Górniak 1986).

Animals poisoned with *P. marcgravii* develop clinical-pathological conditions of acute heart failure, with no evidence of macroscopic changes in the heart due to the rapid progression of the disease (Helayel et al. 2012, Tokarnia et al. 2012, Rodrigues 2015, Nascimento et al. 2018, Serodio et al. 2019). The diagnosis is usually confirmed by identifying characteristic renal lesions through microscopy called hydropic-vacuolar degeneration of the epithelial cells of the distal convoluted urinary tubules, associated with karyopyknosis (Tokarnia & Döbereiner 1986, Tokarnia et al. 2000, 2012, Helayel et al. 2012, Shokry et al. 2017).

The use of cardiac markers, such as cardiac cTnI, has become common in human (Borges et al. 2019) and veterinary (Assis et al. 2017, Cunha et al. 2022) medicine for early detection of myocardial lesions, as they are specific biomarkers for this type of lesion (Fagliari & Thiesen 2015). In a qualitative troponin I (cTnI) analysis, 62.5% of sheep had positive results 4 hours after *P. marcgravii* poisoning, and 100% had positive results 8 hours after poisoning (Cunha et al. 2022). However, despite their clinical importance, information on quantitative serum levels and dynamics of these markers in sheep poisoned with *P. marcgravii* is not found in the literature, raising the possibility of utilizing these markers to detect myocardial lesions before they become evident through conventional microscopy or immunohistochemistry (IHC).

Immunohistochemical techniques with high specificity and sensitivity have been used to detect incipient lesions in the myocardium, mainly with the use of troponin, which is a structural component of cardiac muscle cells released into the bloodstream approximately 3 to 4 hours after the occurrence of cell damage (Aires 1999, Jenkins et al. 2010). Affected myocytes exhibit negative staining, whereas non-necrotic myocytes are positive for troponin (Jenkins et al. 2010). However, information regarding the application of the use of anti-cTnI antibodies for immunohistochemistry is innovative, given that other studies used anti-cTnC antibodies (Pavarini et al. 2012, Costa et al. 2016, Santos et al. 2016, Cid et al. 2021, Pohl 2021), this technique for investigating troponin in sheep poisoned by *P. marcgravii* is not currently found in the literature.

Thus, the objective of this study was to determine the quantitative serum levels of cTnI and evaluate the expression of cTnI, using the immunohistochemical technique, in incipient heart lesions in sheep experimentally poisoned with *P. marcgravii*. Also, to confirm that troponin leakage occurs before morphological lesions in the myocardium become visible through conventional microscopy.

MATERIALS AND METHODS

Animal Ethics. The study was approved by the Ethics Committee on Animal Use (CEUA) under number 7702030518.

Animals. Eight healthy male mixed breed sheep, between five and twelve months of age, were intoxicated with 1g kg^{-1} of freshly collected *Palicourea marcgravii* (Rubiaceae) plants (identified at the "Instituto de Biologia" of the "Universidade Federal Rural do Rio de Janeiro" – UFRRJ, under the code RBR37508), orally administered, following the procedures described by Tokarnia et al. (2012). The animals were continuously monitored, and physical examinations were conducted every two hours. Blood samples were collected at two stages: immediately before plant administration and during the agonizing phase, just before the animal's death. The animals were subjected to necropsy immediately after death, and the lesions were macroscopically evaluated. Fragments from the main organs (heart, lungs, kidneys, liver, spleen, diaphragm, intercostal muscle, adrenal glands, thyroid, and central nervous system) were collected

and preserved in 10% formalin, embedded in paraffin and stained with hematoxylin and eosin (HE).

Serum samples for troponin I analysis. Blood samples were collected from the jugular vein using a 10-millimeter disposable hypodermic needle (size 40×12) and stored in tubes without anticoagulant, which were immediately centrifuged at 700g for 20 minutes. The obtained serum was stored at -20°C for subsequent troponin I (cTnI) level analysis. The serum levels of cTnI were determined using blood samples collected at two different times: at the pre-poisoning moment (T0), immediately before the administration of the toxic plant, and in the agonizing phase, just before the animal's death. The time between ingestion of the toxic plant and death ranged from 5 hours and 56 minutes to 30 hours and 50 minutes. The cTnI levels were measured using the chemiluminescence method through an instrument designed for immunoassays (FineCare Fia Meter FS-112; Celer Biotechnology, Belo Horizonte, Brazil) (Santos et al. 1993).

Cardiac tissue samples for immunohistochemistry. Tissue fragments from the hearts of the eight sheep were subjected to the immunohistochemical technique. Serial sections of the heart were mounted on saline-coated slides. A heart fragment from a sheep without clinical-pathological changes of cardiopathy was used as a negative control, and a heart fragment from an animal intoxicated with an ionophore showing cardiac lesions was used as a positive control. The histological sections were deparaffinized in xylene, rehydrated in alcohol, immersed in two 3% hydrogen peroxide solutions for 15 minutes in each solution to block endogenous peroxidases, and then washed with phosphate-buffered saline (PBS) for two minutes. Subsequently, they were subjected to a water bath in a citrate buffer solution (pH=6) at 98°C for 30 minutes to reactivate antigenic sites. A 5% skim milk (Molico® Nestle Brazil, Sao Paulo, Brazil) was used to block nonspecific reactions. The sections were incubated overnight with an anti-troponin I (anti-cTnI) antibody (Thermo Fisher Scientific®) diluted at 1:100. Subsequently, they were washed with PBS, and a detection system (EnVision FLEX+; Dako®) was used, with a 30-minute incubation in a humid chamber at 37°C. The chromogen was diaminobenzidine (DAB; Dako®), and 1µL of chromogen was diluted to 50µL of substrate buffer (Dako®) for five minutes on each slide. The slides were counterstained with Harris hematoxylin and mounted for visualization under an optical microscope (Eclipse E200, Nikon) and capturing images (MIchrome 6, 6.0 Megapixels). The anti-cTnI antibody used for immunohistochemical analysis was effective in marking sheep myocardium. According to the sheep material used for positive and negative controls in the present study, the cTnI complex is as effective in immunohistochemical analysis as the cTnC complex.

Statistical analysis. The obtained data were evaluated descriptively, and statistical analyses were performed using the Wilcoxon Signed Ranks Test to compare the data from the pre-poisoning phase to those from the post-poisoning phase (agonizing phase, immediately before the animal's death).

RESULTS

Troponin I serum

All sheep in the present study had minimal levels of troponin I (cTnI) before administration of *Palicourea marcgravii* plants and a significant increase in serum levels of cTnI immediately before death (Table 1), indicating leakage due to cardiac injury.

Animals 7 and 8 had the shortest survival time (ST), corresponding to the time between the ingestion of the toxic plant and death: 5 hours and 56 minutes and 8 hours and 23 minutes, respectively; they also had the lowest increase in cTnI level compared to the other animals. However, these levels still indicate the presence of changes and leakage of cTnI from myocytes into the bloodstream. Animal 1 presented the highest serum level of cTnI and the longest ST (29 hours and 50 minutes), followed by Animals 2, 3, 4, 5, and 6 (Table 1).

The results of the first blood collection were lower than 0.001ng mL^{-1}, and a level of 0.001ng mL^{-1} was considered for statistical purposes in the pre-poisoning phase. A significant difference (p<0.05) was found between the data of pre-poisoning and post-poisoning (immediately before death) phases (Table 2).

Table 1. Individual results of quantitative serum analysis of troponin I before and after poisoning (immediately before death) in blood serum samples from sheep poisoned with *Palicourea marcgravii*

	Animal	Before poisoning	Immediately before death	ST	Weight (Kg)	Reference
cTnI (ng mL^{-1})	1	<0.001	17.32	29h 50min	14.5	#
	2	<0.001	11.13	09h 04min	22.1	
	3	<0.001	10.33	16h 02min	21.5	
	4	<0.001	5.73	10h 17min	19.0	
	5	<0.001	4.27	06h 30min	20.5	
	6	<0.001	2.04	21h 07min	16.3	
	7	<0.001	<0.1	05h 56min	15.0	
	8	<0.001	<0.1	08h 23min	27.0	
Mean		<0.001	8.47			
SD			±5.57			

ST = survival time, SD = standard deviation; # No stablished reference range for sheep species; Levels ≥0.001ng mL-1 are considered with presence of changes.

Table 2. Statistical analysis results for data from pre-poisoning and post-poisoning (immediately before death) phases of sheep experimentally poisoned with *Palicourea marcgravii*

Statistical test	Comparison between pre-poisoning and post-poisoning phases
Wilcoxon signed ranks test	0.012*

* Significantly different (p≤0.05).

Immunohistochemical analysis

No areas with loss or decrease in immunoreactivity to the anti-cTnI antibody were found in the heart samples from control animals (Fig.1).

The histological sections of the hearts from the control animals showed immunostaining for the anti-cTnI antibody, confirming the effectiveness of the antibody in marking troponin within the structure of non-injured myocytes (Fig.1-2).

Animal 3 exhibited several groups of cardiac fibers with a moderate to marked decrease in immunoreactivity to the anti-cTnI antibody and several groups of cardiac fibers showing a slight increase in eosinophilia on histopathology (Fig.3-4). Animal 1 showed several areas with a moderate decrease in immunoreactivity to the anti-cTnI antibody and areas with moderate cardiac fibers showing a slight increase in eosinophilia on histopathology (Fig.5-6). Animal 2 exhibited groups of myofibers and isolated myocytes with moderate to marked decrease in immunoreactivity to the anti-cTnI antibody and groups of cardiac fibers showing moderate to marked increase in eosinophilia on histopathology (Fig.7-8).

The immunohistochemical analysis of the animals' hearts showed several groups of myocytes, with a significant decrease or absence of immunoreactivity to the anti-cTnI antibody and individual or grouped myocytes showing loss of immunoreactivity (Table 3). These areas corresponded to the same groups of myocytes in HE that showed slight increases in cellular swelling to increased cytoplasmic eosinophilia and loss of striatum (Fig.3-8). It was possible to notice that several small groups of myocytes that showed a marked decrease in immunoreactivity corresponded to cells with very discrete changes in the HE. It was also observed that the areas with loss of immunoreactivity to the anti-cTnI antibody were larger than the changes noted on routine histological examination.

DISCUSSION

Studies evaluating cardiac markers, such as serum troponin I (cTnI), remain scarce in the veterinary literature; there are only qualitative evaluation data in sheep (Cunha et al., 2022). This seems to be the first study evaluating the quantitative serum cTnI levels in sheep poisoned with *Palicourea marcgravii* and

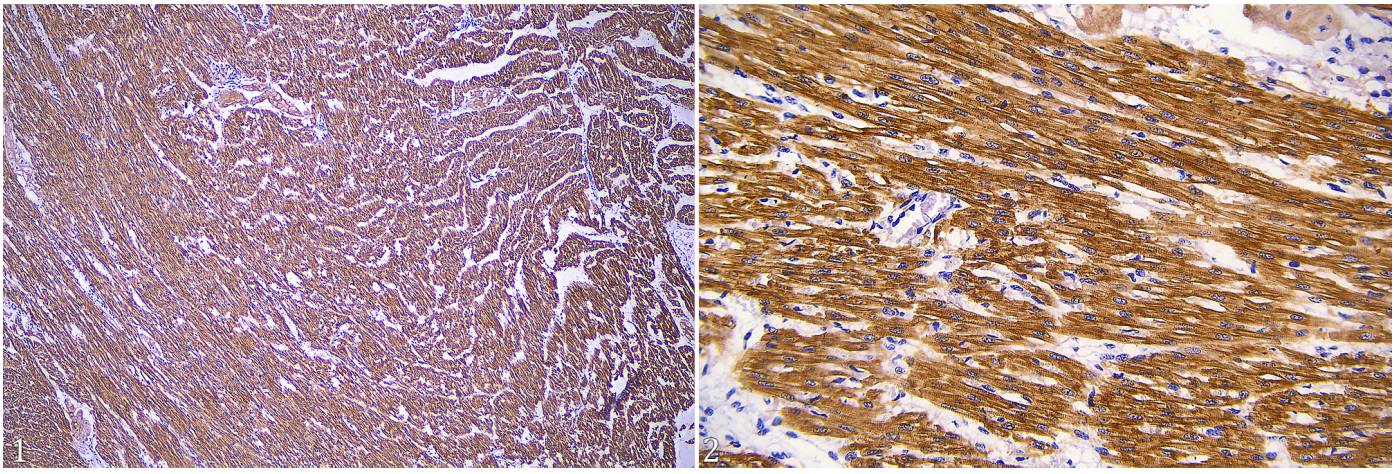

Fig.1-2. Control sheep. Histological section of the heart from a control sheep showing expression of the anti-cTnI antibody. (**1**) IHC, obj.10x. (**2**) IHC, obj.40x.

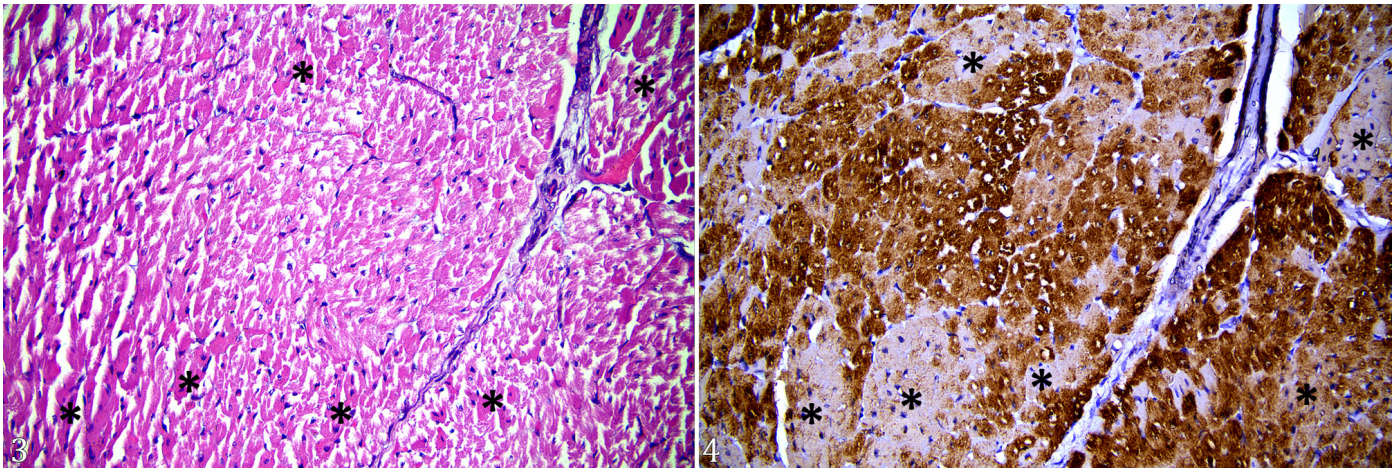

Fig.3-4. Heart of Sheep 3 experimentally poisoned by *Palicourea marcgravii*. (**3**) Several groups of cardiac fibers showing a slight increase in eosinophilia. HE, obj.40x. (**4**) Several groups of myofibers with moderate to marked decrease in immunoreactivity to the anti-cTnI antibody. IHC, obj.40x.

describing the increase of this biomarker in the bloodstream. These biomarkers can assist in the early and *ante mortem* diagnosis of this type of poisoning (Cunha et al. 2022). Additionally, it is the first study to evaluate cardiac tissue damage in these animals through immunohistochemical analysis. This can be an additional tool for confirming the *post mortem* diagnosis, especially in cases where the characteristic renal lesions in poisoned animals are not visualized (Cunha et al. 2022).

Studies reporting the use of anti-cTnI antibodies to detect cardiac changes in sheep are scarce in the literature (Costa et al. 2016, Santos et al. 2016). The human anti-troponin C (anti-cTnC) antibody has been described as sensitive for detecting cardiac lesions in cattle (Pavarini et al. 2012), sheep (Costa et al. 2016, Santos et al. 2016), cats (Cid et al. 2021), and horses (Pohl 2021). The choice of using the anti-cTnI antibody for immunohistochemistry is a novelty

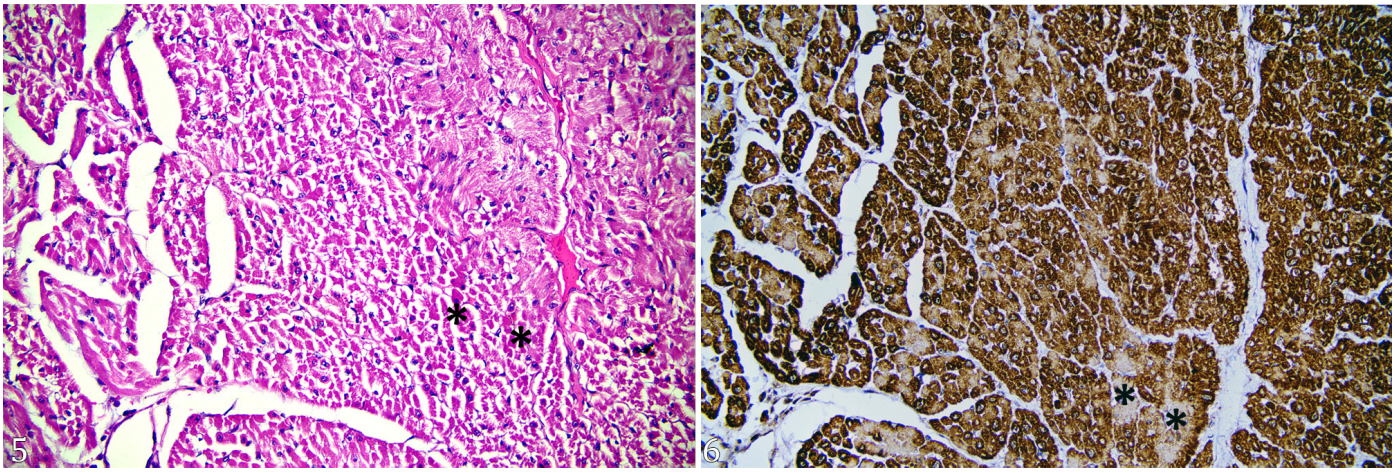

Fig.5-6. Heart of Sheep 1 experimentally poisoned by *Palicourea marcgravii*. (**5**) Areas with moderate cardiac fibers showing a slight increase in eosinophilia. HE, obj.40x. (**5**) Areas with a moderate decrease in immunoreactivity to the anti-cTnI antibody. IHC, obj.40x.

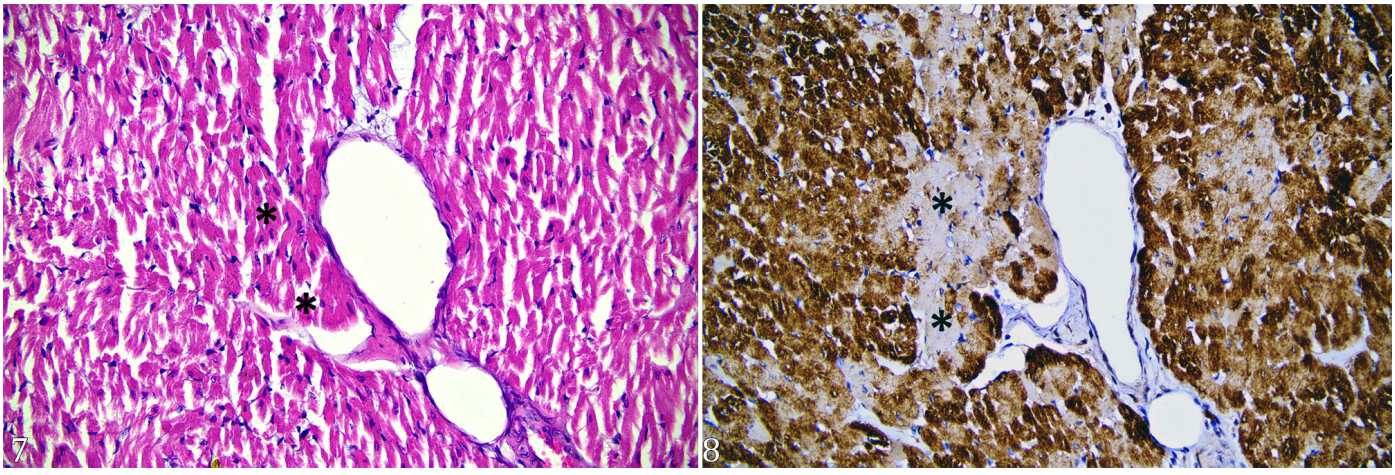

Fig.7-8. Heart of Sheep 2 experimentally poisoned by *Palicourea marcgravii*. (**7**) Cardiac fiber groups showing a slight increase in eosinophilia. HE, obj.40x. (**8**) Groups of myofibers and isolated myocytes with moderate to marked decrease in immunoreactivity to the anti-cTnI antibody. IHC, obj.40x.

Table 3. Individual results of immunohistochemical (IHC) analysis of histological sections of hearts of sheep experimentally poisoned with *Palicourea marcgravii*

Animal	Characteristics of expression of anti-cTnI (IHQ)
1	Extensive areas with moderate to marked decrease in immunoreactivity to the anti-cTnI antibody
2	Groups of myofibers and isolated myocytes with moderate to marked decrease in immunoreactivity to the anti-cTnI antibody
3	Several groups of cardiac fibers with moderate to marked decrease in immunoreactivity to the anti-cTnI antibody
4	Isolated fibers in some areas with a marked decrease in immunoreactivity to the anti-cTnI antibody
5	Small groups of fibers with a moderate decrease in immunoreactivity to the anti-cTnI antibody
6	Few areas with weak or absent immunoreactivity to the anti-cTnI antibody
7	Isolated myofibers with a moderate decrease in immunoreactivity to the anti-cTnI antibody
8	Isolated groups of fibers with a moderate decrease in immunoreactivity to the anti-cTnI antibody

compared to previous studies that utilized the anti-cTnC antibody for the same technique with other plants and adverse situations such as *Amorimia exotropica* poisoning (Pavarini et al. 2012), *Amaranthus spinosus* (Costa et al. 2016), sodium monofluoroacetate (Santos et al. 2016), cardiac injury from kidney disease (Cid et al. 2021), and ionophore antibiotic poisoning (Pohl 2021). The release of cTnI into the bloodstream appears to be associated with irreversible necrotic areas of cardiomyocytes, with the concentration increasing proportionally to the severity of the damage, as seen in studies with other species (Pavarini et al. 2012, Costa et al. 2016, Santos et al. 2016, Cid et al. 2021, Pohl 2021). The largest part of cTnI is found in the contractile apparatus and is released through proteolytic degradation (Collinson et al. 2001). cTnI is released in three forms: free, troponin I and C (TnI-TnC) complex, and troponin T, I and C (TnT-TnI-TnC) complex, which present varying degrees of degradation, with the predominant part of cTnI circulating in a complex form (Oyama & Solter 2004). The anti-cTnC antibody has previously been described as sensitive in detecting cardiac lesions in cattle (Pavarini et al. 2012), sheep (Costa et al. 2016, Santos et al. 2016), cats (Cid et al. 2021) and horses (Pohl 2021).

The poisoning of sheep with *P. marcgravii* caused cardiomyocyte degeneration, resulting in increased serum levels of cTnI, even in animals that did not show pronounced changes in the immunohistochemical analysis. This reinforces the hypothesis that troponin leakage precedes detectable morphological lesions in the myocardium through conventional microscopy and immunohistochemistry. These findings are consistent with some studies in humans, which showed that myocardial necrosis is not required to release cTn. However, cellular death in any form, including autophagy, apoptosis, and necroptosis, can result in cTn release (Wu et al. 2007, Jaffe & Wu 2012).

A significant difference was observed between the baseline levels (pre-poisoning phase) and those obtained immediately before the death of poisoned animals. Serum levels of cTnI below 0.1ng mL^{-1} are considered normal in human medicine (Leal et al. 2003). In contrast, serum levels of cTnI from 0.5ng mL^{-1} are found under conditions of myocardial infarction with cardiomyocyte injury (Leal et al. 2003). Therefore, the cTnI levels in the present study are sufficient to confirm cardiac injury with troponin leakage caused by *P. marcgravii* toxicity.

Regardless of its etiology, damage to the myocardium can result in cTn leakage into the bloodstream. Venkatesan et al. (2020) evaluated serum levels of cTnI in cows with traumatic reticulopericarditis and found increased levels (0.10±0.034ng mL^{-1}) compared to those found in healthy animals (0.0204±0.011ng mL^{-1}). Soares et al. (2019) assessed cTnI levels in 15 cows with clinical ketosis at the beginning of lactation and found significantly higher concentrations of cTnI (0.07±0.03ng mL^{-1}), indicating a high degree of myocardial injury.

The animals in the experiment showed varying degrees of decreased immunoreactivity to the anti-cTnI antibody, confirming the cardiomyocyte injury caused by the experimental intoxication with *P. marcgravii.* These findings indicate that even mild changes in the immunohistochemical analysis are sufficient to indicate cardiac injury with severe troponin leakage.

The increased serum levels of cTnI in the evaluated sheep were caused by its extravasation from cardiomyocytes into the bloodstream due to cell damage. Most of the histological sections of the heart used for immunohistochemistry showed similarity, indicating that the higher the serum levels of cTnI, the lower the immunoreactivity to the antibody. However, this result was not found for Animal 2, denoting no sufficient cardiomyocyte injury in this animal's specific area of heart fragment.

CONCLUSION

The sheep poisoned with *Palicourea marcgravii* presented cardiac lesions that could be detected through analysis of serum levels of cardiac troponin I (cTnI), confirming the hypothesis that leakage of troponin into the bloodstream occurs before morphological lesions in the myocardium can be observed through morphological microscopy and immunohistochemical analysis. The use of the immunohistochemical technique with an anti-cTnI antibody was effective in the early detection of myocardial lesions in sheep experimentally poisoned with *P. marcgravii.*

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Conflict of interest statement.- The authors declare that there are no conflicts of interest.

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