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PATHOLOGY

Intoxication by *Nerium oleander* in cattle: use of immunohistochemistry for troponin C as auxiliary diagnostic method

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ABSTRACT: *Nerium oleander*, also known as "espirradeira" in Brazil, is a shrub or small tree widely used as an ornamental plant in temperate and subtropical regions of the world. Ingestion of *N. oleander* can cause intoxication due to the presence of cardioactive glycosides, in both humans and animals. This study aims to report the epidemiological, clinical, *postmortem*, and immunohistochemical features of an outbreak of natural intoxication by *N. oleander* in cattle and the use of immunohistochemistry (IHC) for troponin C, for the first time, in this intoxication in the species. Twenty-eight cattle had access to pruned branches of *N. oleander* and 20 animals started presenting clinical signs of apathy, anorexia, ataxia, tachypnea, tremors, and recumbency ten hours after the consumption. Eight cattle progressed to death 48 hours after the clinical progression and one was submitted for necropsy. Macroscopic findings included petechiae and ecchymosis on the epicardium and endocardium. Histologically, there were multifocal areas of necrosis of individual or multiple cardiomyocytes, mainly in the left ventricular papillary muscle. Immunohistochemistry for troponin C revealed decrease or absence of cytoplasmic immunolabeling in necrotic cardiomyocytes. Although intoxication by *N. oleander* is uncommon in cattle, it should be considered as a differential diagnosis for other conditions that cause sudden or acute death in livestock and IHC for troponin C is an auxiliary tool in the identification of peracute and acute cardiac lesions.
Key words: toxic plants, espirradeira, cardiotoxic, oleandrin, troponin C.

Intoxicação por *Nerium oleander* em bovinos: uso de imuno-histoquímica para troponina C como método de diagnóstico auxiliar

RESUMO: *Nerium oleander*, conhecido popularmente como "espirradeira" no Brasil, é um arbusto amplamente utilizado como planta ornamental em regiões temperadas e subtropicais do mundo. A ingestão de *N. oleander* pode causar intoxicação devido à presença de glicosídeos cardioativos, tanto em humanos, quanto em animais. Este trabalho tem como objetivo relatar os achados epidemiológicos, clínicos, patológicos e imuno-histoquímicos de um surto de intoxicação natural por *N. oleander* em bovinos e o uso da imuno-histoquímica (IHQ) para troponina C pela primeira vez nesse tipo de intoxicação na espécie. Vinte e oito bovinos tiveram acesso a galhos de *N. oleander* após poda e 20 desses bovinos apresentaram sinais clínicos de apatia, anorexia, ataxia, taquipneia, tremores e decúbito dez horas após a ingestão. Oito bovinos morreram 48 horas após o início do curso clínico e um animal foi submetido à necropsia. Os achados macroscópicos incluíram petéquias e equimoses no epicárdio e no endocárdio. Histologicamente, havia áreas multifocais de necrose de cardiomiócitos individuais ou múltiplos, principalmente no músculo papilar do ventrículo esquerdo. A IHQ para troponina C revelou uma acentuada diminuição ou ausência na imunomarcação citoplasmática em cardiomiócitos necróticos. Embora a intoxicação por *N. oleander* seja incomum em bovinos, ela deve ser considerada como diagnóstico diferencial para outras condições que induzam morte súbita ou aguda em rebanhos e a IHQ para troponina C é uma ferramenta complementar na identificação de lesões hiperagudas e agudas cardíacas.
Palavras-chave: plantas tóxicas, espirradeira, cardiotóxico, oleandrina, troponina C.

Nerium oleander, popularly known as "espirradeira" in Brazil, is a shrub from the Apocynaceae family, native to Asia and the Mediterranean, and it is widely distributed in subtropical and tropical regions (TOKARNIA et al., 2012). In Brazil, *N. oleander* is widely found and used as an ornamental plant in urban neighborhoods (TOKARNIA et al., 2012). *N. oleander* can grow up to four meters in height, has dark-green to gray lanceolate leaves, and, at the end of the branches, has flowers which can be pink, white or red (LANGFORD & BOOR, 1996).

The toxicity of *N. oleander* is attributed to its high concentration of cardenolides, also known as cardioactive glycosides, with oleandrin being the most important of them (PRAVEEN et al., 2012). These glycosides inhibit the sodium-potassium pump in the cytoplasmic membrane of cells, resulting in decreased intracellular potassium levels and calcium accumulation. These alterations impact the contractility and electrical conductivity of cardiomyocytes, leading to acute cardiac insufficiency and eventual death (LANGFORD & BOOR, 1996; SOTO-BLANCO et al., 2006; PEDROSO et al., 2009).

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The cardiac lesions induced by *N. oleander* consumption range from peracute to acute, which may result in minimal or absent cardiac histological lesions (TOKARNIA et al., 2012), requiring the use of complementary diagnostic techniques. Cardiac troponins (cTn) comprising subunits C, T, and I, have high sensitivity and specificity for myocardial injury and are regarded as reliable serum and immunohistochemical biomarkers for detecting early cardiac cell injury in both humans and animals (O'BRIEN, 2008; WELLS & SLEEPER, 2008; PAVARINI et al., 2012; BANDINELLI et al., 2014; ROSSI et al., 2014). The use of the immunohistochemistry (IHC) for troponin subunit C to detect acute cardiotoxic injury in cattle has demonstrated good accuracy (PAVARINI et al., 2012; BANDINELLI et al., 2014).

Although *N. oleander* has low palatability and is an uncommon source of intoxication in livestock, its widespread distribution can predispose to acute and fatal cases of intoxication that represent an important differential diagnosis of acute and sudden death in cattle (LANGFORD & BOOR, 1996; PEDROSO et al., 2009). The aim of this study is to report the epidemiological, clinical, *postmortem*, histological, and immunohistochemical features of a natural outbreak of *N. oleander* intoxication in cattle, with a focus on the use of troponin C marker as an auxiliary diagnostic tool for this intoxication.

An outbreak of intoxication by *N. oleander* in cattle occurred in February 2021 on a farm located in the rural area of Alvorada (29° 59' 24" S 51° 05' 02" W), Rio Grande do Sul state, Brazil. Epidemiological and clinical data regarding the reported outbreak were obtained through an interview with the farm's owner. A total of twenty-eight mixed beef breed cattle of various ages were fed with leaves from pruned branches of *N. oleander*, which were being used as ornamental plants (Figure 1A). Out of the 28 cattle, 20 became ill and eight died (71.4% morbidity and 40% lethality). The eight cattle that died consisted of five males and three females, aged between four and 12-months old. The remaining cattle (20/28) were adults aged between two and 6-year-old.

Ten hours after ingesting the plant, affected cattle started showing clinical signs of apathy, anorexia, ataxia, tachypnea, muscle tremors, and recumbency. The cattle that died had a clinical course of up to 48 hours, while those that recovered had a clinical course of up to 10 days and also exhibited weight loss.

A 12-month-old and mixed breed steer died after a clinical course of 48 hours and was submitted to necropsy. The necropsy was conducted at the Setor de Patologia Veterinária of the Universidade Federal do Rio Grande do Sul (SPV-UFRGS) and sections of main organs (heart, lung, liver, gallbladder, kidneys, urinary bladder, spleen, pancreas, adrenal glands, lymph nodes, central nervous system, pre-stomachs, abomasum, and intestines) were collected, fixed in 10% formalin, and routinely processed for microscopic examination.

The main macroscopic findings were observed in the heart, which included petechiae and ecchymosis in the epicardium and endocardium, mainly in the left ventricle (Figure 1B). Furthermore, the lungs were non-collapsed, heavy, and wet (pulmonary edema), and there was a moderate amount of foamy content in the lumen of the distal third of the trachea and bronchi. In the examination of the rumen, no leaves of *N. oleander* were noted amidst the ruminal content. *Postmortem* examination of the remaining organs was unremarkable.

Histologically, the main alteration was an acute necrotic cardiomyopathy. The cardiac lesions included necrosis of individual or multiple cardiomyocytes (Figure 1C), whith greater prominence in the left ventricular papillary muscle. The cytoplasm of necrotic cardiomyocytes was retracted, hypereosinophilic, with loss of striations and, occasionally, fragmented, while the nucleus was pyknotic or fragmented. The endocardium and epicardium were expanded by multifocal areas of hemorrhage. Pulmonary alveolar edema was also seen. The remaining examined organs did not show any significant microscopic lesions.

Selected sections of myocardium with significant lesions were selected and subjected to IHC using a mouse monoclonal anti-troponin C antibody (1:40, clone 1A2, Novo, Castra, Newcastle, UK). MACH 4 HRP-Universal Polymer (Biocare Medical®, Pacheco, California, USA) was employed as the detection system. The reactions were revealed with chromogen romulin AEC kit (Biocare Medical). Myocardial sections of cattle intoxicated by *Amorimia exotropica* were used as positive controls. Immunolabeling of adjacent normal cardiomyocytes served as internal positive control. The primary antibody was replaced by Universal Negative Control (Biocare Medical®) in negative controls. The IHC protocol for troponin C was modified from previous studies in cattle (PAVARINI et al., 2012; BANDINELLI et al., 2014).

IHC analysis showed marked and multifocal areas of decrease or absence in troponin C expression in the cytoplasm of necrotic cardiomyocytes (Figure 1D). Complete absence of immunolabeling was observed in

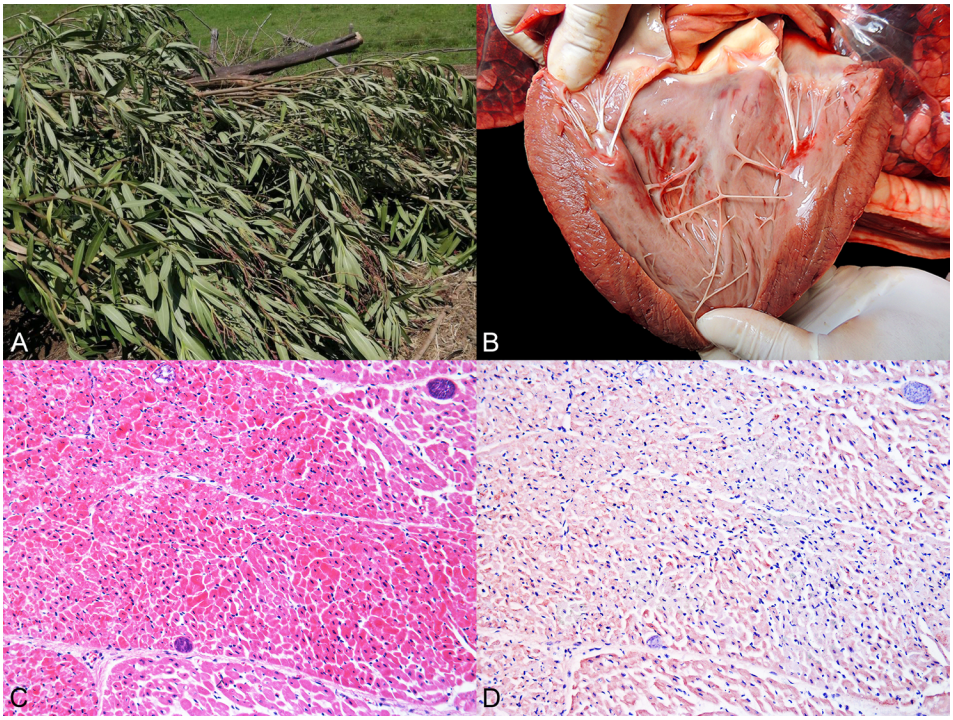

Figure 1 - Intoxication by Nerium oleander in cattle. (A) Pruned branches of N. oleander offered to the cattle. (B) Heart. Multifocal to coalescent areas of hemorrhage in the endocardium of the left ventricle. (C) Heart. Focally extensive area of cardiomyocyte necrosis. Cardiomyocytes exhibit retracted and hypereosinophilic cytoplasm, loss of striations, and fragmented or pyknotic nucleus. HE, 100x. (D) Heart. Multifocal to coalescent absence or decrease of immunolabelling in necrotic cardiomyocytes from figure C. IHC for Troponin C, 100x.

the most severe cardiac lesions based on the histological examination. Anti-troponin C immunolabeling was intense in the cytoplasm of intact cardiomyocytes.

The diagnosis of intoxication by *N. oleander* in cattle in this study was based on epidemiological, clinical, and pathological findings. The history and evidence of consumption of leaves from *N. oleander* branches represented an epidemiological aspect that significantly contributed to the diagnosis of intoxication in our case. In the present study, chemical testing for cardenolides was not available, as it would also be useful to the confirmatory diagnosis. Intoxications by *N. oleander* in cattle are uncommon, and epidemiological factors of natural intoxications include accidental ingestion, after pruning or storms, ingestion of hay contaminated with leaves and branches of *N. oleander*, and food shortage (ASSIS et al., 2010; PEDROSO et al., 2009; TOKARNIA et al., 2012). In our case, the supply of branches that were pruned and placed within reach of the cattle was the cause of the intoxication. This suggests that errors in handling the plants and a lack of knowledge about the toxicity of *N. oleander* were determinant factors in the animals' exposure.

Younger cattle, aged four to 12 months, were more severely affected and the ones that died in this study. It is suggested that younger animals, due to their lower body weight, require a smaller amount of leaves to reach the lethal toxic dose, which is 0.25 to 1.0 g of the plant per kg of body weight (PEDROSO et al., 2009; TOKARNIA et al., 2012). Unfortunately, in this outbreak, it was not possible to measure the amount ingested by each individual animal. However, most toxins produce more severe effects in younger animals because they may lack enzymes needed for biotransformation, and as result, they have a lower capacity to detoxify toxins (OSWEILER, 1996; GÓRNIAK & HARAGUCHI, 2020). Furthermore, data on the age of unaffected animals was not accessible, and such information would have been valuable for the epidemiological investigation during this outbreak.

Most cattle spontaneously intoxicated by *N. oleander* present a peracute to acute clinical course, and death occurs in less than 24 hours (SOTO-BLANCO et al., 2006). However, experimentally poisoned cattle showed a clinical course of approximately 48 hours, as observed in this case, after ingesting a dose of 0.5 mg/kg of the plant (TOKARNIA et al., 1996). This suggests that the clinical course of the intoxication is likely dose-dependent. Furthermore, the severity of the clinical signs and the clinical evolution are directly related to the ingested dose (GALEY et al., 1996; PEDROSO et al., 2009; TOKARNIA et al., 2012).

Clinical manifestations are mainly related to cardiac and digestive disorders (TOKARNIA et al., 1996; SOTO-BLANCO et al., 2006; PEDROSO et al., 2009). In the present outbreak, animals showed nonspecific clinical signs, such as apathy, anorexia, ataxia, tachypnea, muscle tremors, and recumbency. Therefore, the association of pathological and epidemiological data were essential for the diagnosis of *N. oleander* intoxication. Although the morbidity in this outbreak was 71.4% with a lethality of 40%, these rates can vary, and the lethality is usually high (SOTO-BLANCO et al., 2006).

The main cardiac macroscopic findings are nonspecific and included petechiae and ecchymosis in the epicardium and endocardium, which accords with literature descriptions of cattle intoxicated by *N. oleander* (SOTO-BLANCO et al., 2006; PEDROSO et al., 2009; CECI et al. 2020). In some cases, leaves of the plant can be observed in the rumen, allowing for the presumptive diagnosis (SOTO-BLANCO et al., 2006), which did not happen in this case. Furthermore, cattle with sudden death and without previous clinical disease usually do not present macroscopic findings (GAVA et al., 1998). This highlights the importance of collecting organs for histopathological examination, especially the heart, to confirm the diagnosis.

The cardiac histological lesions observed in our study were characteristic of acute necrotic cardiomyopathy of toxic origin and were identical to those described in cattle intoxicated by *N. oleander* (TOKARNIA et al., 1996; PEDROSO et al., 2009; CECI et al., 2020). These cardiac lesions are related to the action of cardiotoxic glycosides present in *N. oleander*, mainly oleandrin, which is readily absorbed after ingestion and reaches the heart, damaging the cardiomyocytes (LANGFORD & BOOR, 1996). Glycosides inhibit the sodium-potassium pump, causing a decrease in intracellular potassium and resulting in the accumulation of calcium, affecting the contractility and electrical conductivity of the heart (LANGFORD & BOOR, 1996; SOTO-BLANCO et al., 2006; PEDROSO et al., 2009). The necrotic cardiac lesions in the cattle of this outbreak were more evident in the papillary muscles, as observed in other conditions that result in acute toxic cardiomyopathy (PAVARINI et al., 2012; BANDINELLI et al., 2014). Myocardial necrosis resulting from toxic insults is more frequently seen in the left papillary muscle and the subendocardial myocardium because these lesions are related to a transitory decrease in vascular perfusion (GALL & CASTILLO-ALCALA, 2022). Therefore, the left papillary muscle corresponds to an important site that should be collected for histological analysis in cases suspected of toxic myocardial injury (BANDINELLI et al., 2014).

IHC for troponin C facilitates the detection of injured cardiomyocytes, as necrotic cells do not show immunolabeling (PAVARINI et al., 2012; BANDINELLI et al., 2014). COSTA et al. (2016) suggest that variations in the intensity of IHC for troponin C may be associated with a peak in troponin release, which occurs in greater amounts between 48-72 hours after cardiac injury (structural reservoir troponin). In the present study, the decrease in the immunolabeling for troponin C was marked and multifocal, which helped to confirm the histological lesions. In addition, the 48-hour clinical progression might be correlated with the pattern of intensity and distribution seen in the IHC (COSTA et al., 2016). Therefore, the use of this exam can assist in the diagnosis of such intoxication in animals. No data were found in the literature regarding the use of IHC for troponin C to identify cardiac lesions in cattle intoxicated by *N. oleander*. However, this technique is not specific to diagnose *N. oleander* intoxications, since it has been used in the diagnosis of *Amorimia exotropica* intoxication in cattle. In those studies, IHC showed well-demarcated areas of decrease or absence expression of troponin C in the cytoplasm of cardiomyocytes, mainly from the left papillary muscle (PAVARINI et al., 2012; BANDINELLI et al., 2014), similarly to what was observed in this study.

The serum activity of the cardiac enzyme creatine kinase (CK-MB) can be considered a marker of myocardial injury in humans and animals. However, serum cardiac troponins have been used and demonstrate good reliability. Cardiac troponins I (cTnI) and T (cTnT) are released from injured cardiomyocytes and can be detected in the blood. These cardiac troponins are well-conserved between species, so immunoassays designed for humans can be effectively used in other species, including dogs, cats, horses, and cattle (ALLISON, 2022). In our

study, the detection of CK-MB, cTnI and cTnT in the blood of cattle showing clinical signs would have been helpful in the presumptive diagnosis of acute myocardial injury caused by *N. oleander*. However, blood samples from the affected animals were not available for this purpose.

Intoxication by *N. oleander* in cattle must be differentiated from cases of sudden death and from conditions that present similar clinical signs to those observed in this study. Among these conditions, intoxication by acute cardiotoxic plants, such as *Amorimia exotropica* (PAVARINI et al., 2011) and *Palicourea marcgravii* (TOKARNIA et al., 2012), is considered to be the main differential diagnosis. Diseases that present peracute to acute clinical evolution, such as nitrate and nitrite poisoning (JÖNCK et al., 2013), intoxication by plants containing cyanogenic glycosides (GAVA et al., 1992; JUFFO et al., 2012; GRIS et al., 2021), fulguration (WATANABE et al., 2010), intoxication by acute hepatotoxic plants (TOKARNIA et al., 2012), and intoxication by ionophores (NOGUEIRA et al., 2009), should also be considered as differential diagnoses. However, these conditions have distinct epidemiological findings. In addition, the history and evidence of consumption of the plant by the cattle in this study were essential for the accurate diagnosis.

Although intoxication by *N. oleander* in livestock animals is uncommon, this ornamental plant should be considered as a differential diagnosis of conditions that cause sudden or acute clinical disease and histological lesions of acute cardiomyocytes necrosis. Additionally, IHC for troponin C can be used as an auxiliary test to diagnose intoxication by *N. oleander*. Intoxications by *N. oleander* occur, mainly, due to management errors, combined with a lack of knowledge about its toxicity. Hence, it is important that veterinarians inform livestock handlers and farm owners about this intoxication and guide them on how to prevent it, thus avoiding the death of animals and subsequent economic losses.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL DECLARATION

We, the authors of the article entitled "Intoxication by *Nerium oleander* in cattle: use of immunohistochemistry for troponin C as auxiliary diagnostic method", declare, for all due purposes, that this project has not been submitted for evaluation to the Ethics Committee of the University /Research Institute "Comissão de Ética no Uso de Animal - CEUA - UFRGS", but we are aware of the content of the Brazilian resolutions of the National Council for Control of Animal Experimentation - CONCEA "http://www.mct.gov.br/index.php/content/view/310553.html" if it involves animals. Thus, the authors assume full responsibility for the presented data and are available for possible questions as required by the competent authorities.

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