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Original Article
Livestock Diseases

Crotalaria spectabilis Roth. (Fabaceae, Papilionoideae) seed poisoning in cattle and use of liver biopsy to diagnose subclinical disease[1]

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ABSTRACT.- Cavasani J.P.S., Dias L., Santos I.G., Garcia D.M., Silva W.D.M., Ferreira D.L., Colodel E.M. & Furlan F.H. 2024. ***Crotalaria spectabilis* Roth. (Fabaceae, Papilionoideae) seed poisoning in cattle and use of liver biopsy to diagnose subclinical disease.** *Pesq. Vet. Bras 44:e07439, 2024*. Laboratório de Patologia Veterinária, Hospital Veterinário, Universidade Federal de Mato Grosso, Av. Fernando Corrêa da Costa 2367, Boa Esperança, Cuiabá, MT 78060-900, Brazil. E-mail: fernando.furlan@ufmt.br

Crotalaria spectabilis seed poisoning has been reported in cattle on rural properties in Diamantino and Campo Verde municipalities in Mato Grosso, Brazil. The disease appeared 75 to 120 days after the animals were fed a diet contaminated with *C. spectabilis* seeds. Clinically, the animals showed a lack of appetite, progressive weight loss, apathy, photosensitivity, lateral recumbency, and death. Some animals developed neurological manifestations as a consequence of hepatic encephalopathy. In total, 37 cattle died, and six cattle were necropsied. The main macroscopic changes consisted of ascites and a firm and decreased liver with an irregular surface and multifocal pale yellowish discoloration. Microscopically, hepatocyte loss with fibrosis, megalocytosis and bile duct proliferation was observed. Animals with severe hepatic damage also had *status spongiosus* in the central nervous system. Furthermore, a hepatic biopsy of 15 cows who consumed contaminated grains but did not develop clinical disease revealed histologic liver changes similar to necropsied animals.

INDEX TERMS: *Crotalaria spectabilis*, hepatic fibrosis, pyrrolizidine alkaloids, monocrotaline, cattle.

RESUMO.- [Intoxicação por sementes de *Crotalaria spectabilis* Roth. (Fabaceae, Papilionoideae) em bovinos e a utilização de biópsia hepática para diagnosticar doença subclínica.] Relata-se dois surtos de intoxicação por sementes de *Crotalaria spectabilis* em bovinos em propriedades rurais nos municípios de Diamantino e Campo Verde, Mato Grosso, Brasil. A doença apareceu 75 a 120 dias depois que os animais foram alimentados com uma dieta contaminada com sementes de *C. spectabilis*. Clinicamente, foi observado falta de apetite, perda de peso progressiva, apatia, fotossensibilização, decúbito lateral e morte. Alguns animais desenvolveram manifestações neurológicas secundárias à encefalopatia hepática. No total, 37 bovinos morreram e seis deles foram submetidos à necropsia. As principais alterações macroscópicas consistiram em ascite e fígado diminuído em tamanho com superfície capsular irregular, difusamente firme e com áreas multifocais amarelo-pálidas. Microscopicamente, foi observado perda de hepatócitos acompanhada de fibrose hepática com megalocitose e proliferação de ductos biliares. No sistema nervoso central de bovinos com lesões hepáticas graves havia *status spongiosus*. A biópsia hepática de 15 bovinos que consumiram grãos contaminados, mas estavam clinicamente sadios, revelou alterações hepáticas similares às descritas nos animais submetidos à eutanásia.

TERMOS DE INDEXAÇÃO: Crotalária, fibrose hepática, alcaloides pirrolizidínicos, monocrotalina, bovinos.

INTRODUCTION

The use of *Crotalaria* spp. is associated with its ability to mitigate the erosive effect on the soil, raise soil fertility, fixation of nitrogen, and in the fight against nematodes in

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the ground, in consortium or covering plants (Pacheco & Silva-López 2010).

The genus contains approximately 690 species found worldwide, many poisoning farm animals (Tokarnia et al. 2012, Burrows & Tyrl 2013). This genus has fourteen species in Brazil, including *C. juncea* and *C. mucronata* (= *C. pallida*) associated with lung alterations (Nobre et al. 1994, Lemos et al. 1997, Boghossian et al. 2007, Pessoa et al. 2013, Borelli 2015, Borelli et al. 2016), while *C. retusa, C. incana* and *C. spectabilis* are related to toxic hepatopathy (Becker et al. 1935, Sanders et al. 1936, Piercy & Rusoff 1946, Emmel 1948, Nobre et al. 2004a, 2004b, 2005, Queiroz et al. 2013, Ribeiro 2020).

The main active principle of the hepatotoxic species of the genus *Crotalaria* is a phytochemical called monocrotaline, a hepatotoxic pyrrolizidine alkaloid (HPA) (Scupinari et al. 2020). After ingestion, the organism excretes much of the monocrotaline. However, the non-excreted fraction is bioactivated in the hepatocytes by the action of the cytochrome P450 enzymatic complex through its oxidation, forming a highly reactive pyrrole species called dehydromonocrotaline (Santos et al. 2008), which binds permanently to molecules of deoxyribonucleic acid (DNA) compromising protein synthesis and cell division (Honório Junior et al. 2010, Moreira et al. 2018).

Crotalaria spectabilis (Leg. Papilonoidae) is one of 690 species in this genus, with limited distribution to tropical and subtropical zones (Mosjidis & Wang 2011). Seeds of *C. spectabilis* have been reported to cause poisoning in pigs (Ubiali et al. 2011) and horses in Brazil (Lacerda et al. 2021, Milani et al. 2021). This study reports two outbreaks of chronic poisoning caused by seeds of *C. spectabilis* in cattle.

MATERIALS AND METHODS

Animal Ethics. This study was conducted following the criteria established by the Ethics Committee for the Use of Animals (CEUA) of the "Universidade Federal de Mato Grosso" (UFMT) under protocol No. 23108.918801/2017-87.

Epidemiological investigation and clinical data. Two properties in Mato Grosso, Brazil, where the disease was reported, were visited: one in the municipality of Diamantino (Property A) and another in Campo Verde (Property B). During technical inspections, epidemiological and clinical data were obtained through interviews with the owners, veterinary doctors, and farm employees.

Necropsy, biopsy, and histopathologic evaluation. Two sick cattle were euthanized in extremis and necropsied at Property A (Bovine 1 and 2), and four that died spontaneously were necropsied at Property B (Bovine 3 to 6). Fragments of organs were collected and fixed in 10% formalin.

Additionally, a percutaneous liver biopsy was performed on the remaining 15 cows of Property B. The point of choice for the needle introduction was the 11th right intercostal space, approximately 20cm below the back line, at the intersection of an imaginary line between the tuber coxae and the scapula and another line perpendicular to the 11h intercostal area; this point corresponds to the topographic position of the right lobe of the liver. After trichotomy, local anesthesia was performed by infiltration block with 2% lidocaine without vasoconstrictors, followed by field antiseptic with iodized alcohol. Subsequently, the biopsy needle was inserted into the liver by percutaneous and transthoracic access (Braga et al. 1985, Barros et al. 2007). The fragment of the liver was collected and fixed in 10% formalin.

Fragments of all organs of the necropsied animals and the liver biopsies were processed according to histological routine, embedded in histological paraffin, cut in 5μm, and stained with hematoxylin-eosin (HE). Additionally, histological liver cuts of all animals were submitted to Masson's Trichrome staining (Prophet et al. 1992).

The liver biopsy evaluation was performed according to Barros et al. (2007). where cows were considered "positive" if examination of the liver fragments revealed at least two of these three changes: hepatomegalocytosis, fibrosis and bile duct hyperplasia. Cows whose livers did not present at least two lesions were considered "negative".

Evaluation of contaminated material. Samples of the residues used in animal feeding at Property B were collected for qualitative assessment of the presence of seeds of *Crotalaria* spp.

Plant identification. Viable seeds that contaminated the corn residues were planted. After germination and growth, samples of the plants containing flowers were collected in exsiccate and sent to the Central Herbarium of the UFMT for identification.

RESULTS

Epidemiological data

On Property A, the disease occurred from October to November 2020. This property was dedicated to beef cattle farming in the crop-livestock integration system. The owner reported he planted winter corn (*Zea mays*) intercropped with *Crotalaria spectabilis* to combat nematodes and incorporate nitrogen in the soil. Part of the July 2020 harvest could not be sold due to contamination of the grains with *C. spectabilis* seeds. Approximately 2,000 cattle were being fattened at the time, and all the contaminated corn was used as complementary food for these animals, which consumed it in around five days. The owner could not provide information on the amount of corn contaminated with *C. spectabilis* seeds fed to the animals or the level of contamination. Twenty cattle became ill, and all of them died four to six months after consuming the contaminated corn (1% morbidity, 1% mortality, 100% lethality), and two were necropsied (Bovines 1 and 2).

The disease occurred from October 2020 to January 2021 on Property B, which is dedicated to dairy farming. According to the owner, for approximately 90 days, 32 dairy cows and two horses, all kept in paddocks with *Panicum maximum*, were supplemented with 5kg/animal/day of residues from cleaning and drying corn grains purchased in the region. Analysis of a 275g sample of corn residue fed to the animals showed 25g (9.1%) of *Crotalaria* sp. seeds. (Fig.1). The animals consumed approximately 454g of *Crotalaria* sp. seeds during this period. Dietary supplementation began at the end of July 2020, and the first deaths occurred in mid-October 2020. Seventeen cows (morbidity and mortality 53%, lethality 100%) and two horses died (morbidity, mortality, and lethality 100%), and, among these, four cows were necropsied (Bovines 3-6). The seeds that contaminated the corn residue were identified as *C. spectabilis*.

Clinical signs and postmortem evaluation

Clinical signs appeared approximately 75 and 120 days after the beginning of consumption on Properties B and A, respectively. The clinical course of the disease was chronic on both properties, ranging from 45 to 94 days. The clinical signs observed in cattle from the outbreaks were similar, starting with inappetence and progressive weight loss and progressing to apathy, separation from the herd, diarrhea,

tenesmus, and dry feces. Subsequently, muscle tremors and sternal recumbency progress to lateral recumbency and death. Two horses from Property B showed neurological signs, including blindness and collisions with obstacles, depression, aggression, and motor incoordination. These manifestations were also observed in Bovines 4 and 5. Additionally, Bovine 1 showed photosensitivity characterized by tearing and increased volume in the submandibular and dewlap regions. (Fig.2). Table 1 summarizes the period of consumption, days from the beginning of consumption to first clinical signs, clinical course, days from the beginning of consumption to clinical outcome, and clinical outcome of the disease on the necropsied cattle.

At *post mortem* examination, all the cattle presented mild ascites and reduced liver size, with an irregular, diffusely firm surface, with multifocal to coalescent areas of pale yellowish discoloration (Fig.3). The same changes were observed on the cut surface (Fig.4).

In the histopathological study of Bovines 1-6, the primary lesions were observed in the liver. They were characterized by varying degrees of disorganization of the hepatic cord

Table 1. *Crotalaria spectabilis* seed poisoned bovines according to property, period of consumption, days from the beginning of consumption to first clinical signs, clinical course, days from the beginning of consumption to clinical outcome and clinical outcome of the disease on the necropsied cattle

Bovine number/ Property	Consumption period	Days from the beginning of consumption to the first clinical signs	Clinical course of the disease	Days from the beginning of consumption to clinical outcome	Clinical outcome
1/A	5 days	120 days	45 days	165	E*
2/A	5 days	120 days	45 days	165	E
3/B	90 days	75 days	65 days	140	ND**
4/B	90 days	75 days	77 days	152	ND
5/B	90 days	75 days	92 days	167	ND
6/B	90 days	75 days	94 days	169	ND

* E = euthanasia, ** ND = natural death.

Fig.1-4. *Crotalaria spectabilis* poisoning in cattle. (**1**) Cleaning and drying wastes of corn grains contaminated with *C. spectabilis* seeds used in animal feeding of cattle of Property B. Inset: Note the seeds of *C. spectabilis* (Black seeds). (**2**) Bovine 1 showing increased volume in the submandibular and dewlap regions (edema). (**3**) Bovine 2. The liver is firm and of reduced size, with an irregular surface and multifocal to coalescent areas of pale discoloration. (**4**) Cut surface of the liver of Bovine 2. There are multifocal to coalescent pale discolored areas.

and replacement of the liver parenchyma by multifocal to coalescent proliferation of fibrous tissue with proliferation of bile ducts. Megalocytosis was frequent, evidenced by hepatocytes with a large nucleus with a vesicular appearance (with chromatin concentrated on the periphery and visible nucleolus) and abundant cytoplasm (Figs.5 and 6). Masson's trichrome staining revealed significant collagen deposition in these areas (Fig.7).

Furthermore, in Bovines 2-5, moderate to severe vacuolation of the cerebral white matter (*status spongiosus*) was observed, mainly at the junction between the white matter and gray matter (Fig.8). Sometimes, in the gray matter, groups of Alzheimer type II astrocytes, characterized by enlarged cells with pale nuclei and vesicular chromatin, were observed.

After the diagnosis of liver fibrosis, our research team returned to Property B in November 2020 and performed a liver biopsy on 15 cows (Bovines 7-21) that were clinically healthy but had consumed corn residue contaminated with Crotalaria *spectabilis* seeds. This analysis revealed histological changes in 12 cows. There were varying degrees of fibrosis with periportal or multifocal to coalescent distribution, in addition to megalocytosis and bile duct proliferation (Fig.9). Histological changes of all cattle are summarized in Table 2. There were five cows positive and ten negative. In January 2021, after one of the positive cows fell ill and died (Bovine 9), the owner decided to sell the positive cows that showed a higher degree of liver fibrosis at the hepatic biopsy (Bovines 11 and 15). The remainder of the herd was monitored until July 2022, and no more sick cows were found.

DISCUSSION

The diagnosis of *Crotalaria spectabilis* seed-induced poisoning was based on epidemiological data, clinical signs, *post mortem* and histopathological findings, and the presence of seeds of *C. spectabilis* along with corn grains used for animal feeding.

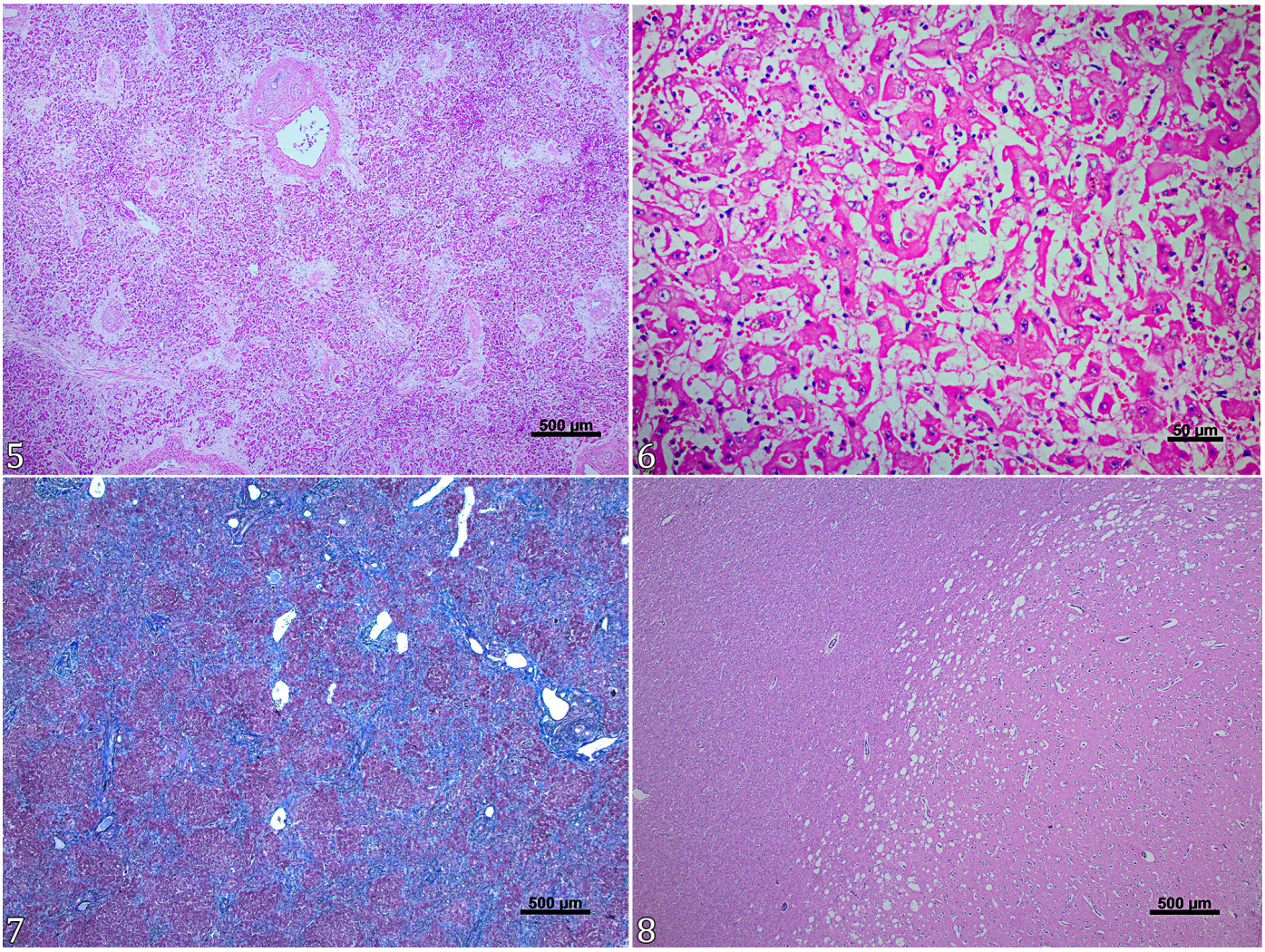

Fig.5-8. Histologic changes observed in *Crotalaria spectabilis* poisoning in cattle (**5**) Bovine 4. The liver sinusoids are disorganized with hepatocyte loss and multifocal to coalescent proliferation of fibrous connective tissue replacing the hepatic parenchyma. HE, obj.2.5x. (**6**) Liver of Bovine 2. There is severe hepatocyte cord disorganization with proliferation of fibrous connective tissue and megalocytosis. HE, obj.20x. (**7**) Liver of Bovine 2. There is a marked proliferation of fibrous connective tissue with the production of collagen (marked in blue), replacing the hepatic parenchyma. Masson's Trichrome, obj.2.5x. (**8**) Cortex of Bovine 4. There is severe vacuolization in the transition region between the white and the gray matter (status spongiosus). HE, obj.2.5x.

The use of *Crotalaria* sp. species in Brazilian agriculture has long been expanding and is associated with its ability to fix nitrogen and control nematodes in the soil (Pacheco & Silva-López 2010). Animals generally consume the plant during pasture, when the plant invades the pasture (Sanders et al. 1936, Piercy & Rusoff 1946, Nobre et al. 2005, Fletcher et al. 2011, Riet-Correa et al. 2011, Botha et al. 2012, Maia et al. 2013, Queiroz et al. 2013) or accidentally, when seeds of *Crotalaria* spp. contaminate animals food (Hooper & Scanlan 1977, Ubiali et al. 2011, Lacerda et al. 2021, Milani et al. 2021), as observed in this study.

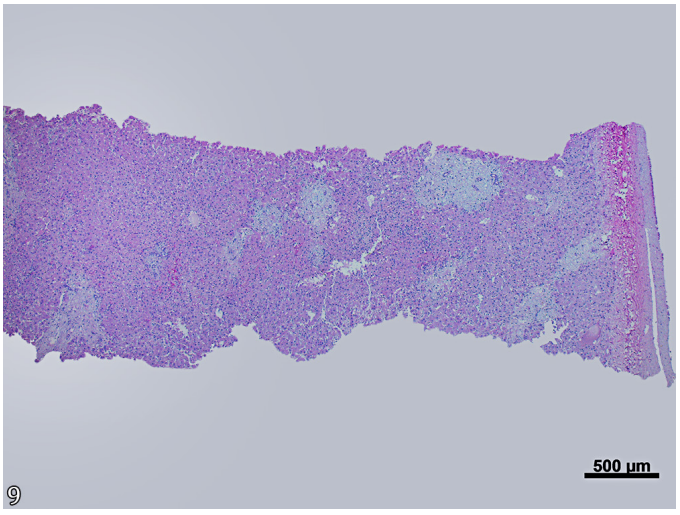

Fig.9. Poisoning by *Crotalaria spectabilis*. Percutaneous liver biopsy of Bovine 9. There is multifocal proliferation of fibrous connective tissue replacing the hepatic parenchyma. HE, obj.2.5x.

In Brazil, there are few reports of *Crotalaria* species causing hepatic injury in animals, except *C. retusa*, which is described as poisoning cattle, sheep, goats and horses in various states of the northeast region (Nobre et al. 2004a, 2004b, 2005, Silva et al. 2006, Assis et al. 2009, Riet-Correa et al. 2011, Maia et al. 2013, Geraldo Neto et al. 2013). *C. incana* was described as affecting cattle in southern (Paraná state) (Queiroz et al. 2013), and *C. spectabilis* seeds were reported in the central-western region, responsible for causing poisoning in pigs in Mato Grosso (Ubiali et al. 2011) and in horses in the Distrito Federal and Goiás (Lacerda et al. 2021, Milani et al. 2021), while Ribeiro (2020) reported the disease in cattle pasturing green leaves and pods in Mato Grosso.

The clinical and pathological changes observed in this report are similar to those described in cases of chronic liver injury in cattle caused by *C. spectabilis* (Becker et al. 1935, Sanders et al. 1936, Piercy & Rusoff 1946, Emmel 1948, Ribeiro 2020), *C. retusa* (Nobre et al. 2004b), and other plants containing HPAs as active substances, such as *Senecio* spp., *Echium* spp., *Cynoglossum* spp., *Amsinckia* spp., and *Heliotropium* spp. (Méndez et al. 1985, Baker et al. 1991, Stegelmeier et al. 1996, Lucena et al. 2010, Stegelmeier 2011, Shimshoni et al. 2015, Panziera et al. 2018). However, in Brazil, only *Crotalaria* spp., *Senecio* spp., and *Echium* spp. are related to affecting animals.

As observed in this study, clinical symptoms or death of cattle grazing HPAs containing plants may occur after a latent period of months or years after the last intake of the green plant and seeds or after the plant has wilted and is no longer evident in the pasture (Gilruth 1903, Sanders et al. 1936, Piercy & Rusoff 1946, Tokarnia & Döbereiner 1984, Méndez et al. 1985, Barros 2016). The clinical manifestations of HPAs poisoning in cattle are secondary to chronic hepatic lesions (Gilruth 1903, Sanders et al. 1936, Piercy & Rusoff

Table 2. Histopathological findings in *Crotalaria spectabilis* seeds poisoning in cattle

Bovine/Property	Liver fibrosis	Hepatomegalocytosis	Biliary duct proliferation	Status spongiosus	Liver biopsy
1/A	+[a]	++	-	-	
2/A	+++	+++	++	+	
3/B	+++	++	++	+	
4/B	+++	+++	++	+++	
5/B	+++	+++	++	++	
6/B	+++	+++	++	-	
7/B	-	+	-	-	Negative
8/B	+	+	++	-	Positive
9/B	++	++	++	-	Positive
10/B	-	+	-	-	Negative
11/B	++	+	++	-	Positive
12/B	-	+	-	-	Negative
13/B	-	+	-	-	Negative
14/B	-	++	-	-	Negative
15/B	++	+++	++	-	Positive
16/B	-	+	-	-	Negative
17/B	+	++	-	-	Positive
18/B	-	+	-	-	Negative
19/B	-	-	-	-	Negative
20/B	-	-	-	-	Negative
21/B	-	-	-	-	Negative

+ mild, ++ moderate, +++ severe, - no lesions.

1946, Emmel 1948, Tokarnia & Döbereiner 1984, Méndez et al. 1985, Barros 2016, Ribeiro 2020). Acute disease cases are rare and restricted mainly to experimental trials (Emmel 1948, Tokarnia et al. 2012). Repeated ingestion of small doses of green plants or seeds over a prolonged period causes progressive liver injury over weeks or months until the lesion becomes sufficiently severe and signs of liver failure ensue. In such cases, cattle ingest sublethal doses insufficient to induce the acute form. However, small portions can produce negligible lesions that, over extended periods, can merge into significant chronic injuries that result in clinical signs of liver failure (Tokarnia & Döbereiner 1984).

The classic lesional pattern caused by plants containing HPAs (hepatocyte loss, fibrosis and increase in the whole volume of the hepatocyte [nucleus and cytoplasm]) has been described since the end of the 19th century. In Germany, it was observed in Schweinsberg disease (Schweinsbergerkrankheit) (Friedberger & Fröhner 1889, Meyers 1909), in Canada in Pictou cattle disease (Johnston 1892) and in New Zealand in Winton's disease, illnesses caused by the consumption of *Senecio jacobaea* (Gilruth 1903, Derbyshire 2010, Petzinger 2011), in the same way as Bottom disease (Schroeder 1893) described in the USA and Kimberley horse disease or Walk-about disease described in Australia, both caused by the consumption of *Crotalaria* spp. (Millar 1899, Murnane & Ewart 1928). Although these diseases' clinical and lesional patterns had been characterized at that time, their etiology remained unknown. It was Gilruth (1903) who, for the first time, experimentally reproduced these lesions by feeding animals with a plant (*S. jacobaea*), but its active principle remained a mystery. Previous studies by Grandval & Lajoux (1895) had already detected HPAs in plants. However, the researchers conducted by Watt (1909) suggested, for the first time, that these compounds could cause disease, and Cushny (1911) experimentally confirmed this hypothesis in laboratory animals.

Monocrotaline is the most common pyrrolizidine alkaloid in the hepatotoxic *Crotalaria* species and primarily has hepatotoxic effects (Honório Junior et al. 2010, Burrows & Tyrl 2013). As the hepatic cytochrome P450 system is the main responsible for the production of these toxic pyrroles, the liver is the primary target organ (Sandini et al. 2013). Pyrroles injure hepatocytes, irreversibly binding to DNA (the alkylating effect) and inhibiting hepatocyte mitosis (Cheeke 1988). The DNA continues to be synthesized in the nucleus, and the nuclear and cytoplasmic volumes of the non-dividing cell continue to increase (Prakash et al. 1999). These cells with increased volume of cytoplasm and nucleus, noted in this study, have already been interpreted by some authors as embryonic cells (Gilruth 1903) or hypertrophy of hepatocytes and their nuclei (Salmon 1897, Harris et al. 1942a, 1942b). On the other hand, Bull (1955), referred to these cells using the term megalocytosis (used to this day) and correctly suggested that their origin is due to the direct action of HPAs. Despite their volume, hepatocytes are not higher functioning cells; their metabolism diminishes considerably (Seawright et al. 1991), and those attempting mitosis die (McLean 1970) and are replaced by connective fibrous tissue. These changes are referred to as hepatic megalocytosis with hepatocyte loss and fibrosis, respectively. Liver failure occurs when the lesion extends beyond 70-80% of the parenchyma, resulting in the animal's death (Cullen & Stalker 2007). A portion of the pyrroles may escape into the general circulation (spillover effect), causing damage to other tissues, such as those of the kidney and lungs, as cytochrome P450 enzymes are also present in these organs (McLean 1970, Molyneux et al. 2011), changes not observed in this study.

Clinical and morphological changes consistent with liver encephalopathy were observed in cattle with the most severe liver damage (Bovines 2-5). This change is due to increased serum ammonia due to failure in liver metabolism. The astrocytes metabolize the excess of ammonia in the systemic circulation, forming glutamine, which induces changes in osmoregulation of the central nervous system, promoting the formation of intermingling edema and causing vacuolization principle in the white substance (Cantile & Youseff 2007). Additionally, two horses from Property B showed neurological and clinical signs similar to Bovine 2-5 but were not necropsied, as the deaths were not notified to veterinarians. According to the literature, horses poisoned with *C. spectabilis* show predominantly neurological signs (Lacerda et al. 2021, Milani et al. 2021).

Five out of the 15 cattle submitted to liver biopsy showed typical lesions of HPA poisoning (Sanders et al. 1936, Nobre et al. 2004a, 2004b, Barros et al. 2007, Panziera et al. 2018). However, after the death of Bovine 9, the owner sold the other two positive cows with higher degrees of liver fibrosis (Bovines 13 and 17), which did not allow the evaluation of the sensibility of the test. The test's specificity was high (100%) once none of the negative cows died during the observation period (November 2020 to July 2022) following the hepatic biopsy. Barros et al. (2007), about the high sensitivity and specificity of liver biopsy for the detection of subclinical cases of *Senecio brasiliensis* poisoned cattle (animals clinically healthy but with hepatic fibrosis), conclude that this technique is an adequate tool for minimizing productive losses caused by PAs in cattle because these animals could be slaughtered before they present the clinical disease. None of the cows had any negative effect related to the liver biopsy technique observed. Two cattle considered positive on liver biopsy remained clinically normal 20 months after the biopsy, and this leads to the conclusion that not all cases that have *C. spectabilis* poisoning lesions on biopsy develop liver failure. Similar recovery cases have already been reported for *S. brasiliensis* (Barros et al. 2007).

CONCLUSIONS

The consumption of grains contaminated with *Crotalaria spectabilis* seeds caused cattle death in this study. This disease is a chronic toxic hepatopathy characterized by megalocytosis with hepatocyte loss and fibrosis. In cases of severe liver injury, there are neurological clinical signs, with status spongiosus in the central nervous system.

Percutaneous liver biopsy can be an essential predictive mechanism in subclinical cases of bovines suspected of *C. spectabilis* poisoning.

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

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