Clinical and laboratory evaluation of sheep experimentally intoxicated with *Crotalaria spectabilis* (leg. papilionoidea) seeds

Evidências clínico-patológicas de ovinos intoxicados experimentalmente com sementes de Crotalaria spectabilis *(leg. papilionoidea)*

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Diana Consuelo Cifuentes Sanchez[a], Kalina Mária de Medeiros Gomes Simplício[a], Lucimara Antonio Borges[a], José Jurandir Fagliari[b], Julio Carlos Canola[b], Mário Roberto Hatayde[c]

[a] Veterinarian, Doctoral student in Veterinary Medicine, School of Agrarian and Veterinary Science (Unesp), Jaboticabal, SP - Brazil, e-mail: dccs111@hotmail.com, kalinamms@hotmail.com, luci.borges95@hotmail.com
[b] Veterinarian, PhD in Veterinary Medicine, professor at the School of Agrarian and Veterinary Science (Unesp), Jaboticabal, SP - Brazil, e-mail: fagliari@fcav.unesp.br, canola@fcav.unesp.br
[c] Veterinarian, Doctorate in Clinical Medicine, professor at the School of Agrarian and Veterinary Science (Unesp), Jaboticabal, SP - Brazil, e-mail: hatayde@fcav.unesp.br

Abstract

The objective of the present study was to evaluate the clinical and laboratory alterations and hepatic lesions caused by the intake of *Crotalaria spectabilis* in sheep. Fifteen ewes (unknown breed) were used, which were allocated into three groups of 5 animals. These groups received milled *C. spectabilis* seeds in their feed over 28 days, with inclusion rates of 0.4% (G1), 0.6% (G2) and 1% (G3). Blood samples and hepatic biopsies for histology were drawn at: T0 (day before the seeds were included in the diet), T1 (7 days after the seeds were included), T2 (14 days), T3 (21 days) and T4 (28 days). The results were submitted to variance analysis (ANOVA) using Statistical Analysis System software (SAS), and differences between the means were compared by the Tukey posttest ($p < 0.05$). Only one animal from G3 presented clinical signs of intoxication at T3. The results from the hematological parameters were within the normal range for this species. Serum activity of aspartate aminotransferase (AST) and creatine phosphokinase (CK) did not show significant differences among the groups, whereas gamma-glutamyltransferase (GGT) activity was significantly different between G1 (33.66 ± 25.8 U/L) and G2 (67.3 ± 14.8 U/L) at T0. Ultrasonography evaluation revealed hepatic alterations throughout the experimental period, which was confirmed by histology. Therefore, analyses of hemogram and hepatic function alone do not have diagnostic value for *C. spectabillis* intoxication in sheep, and it is necessary to perform additional tests, such as ultrasonography and percutaneous liver biopsies.

Keywords*:* Hepatotoxic plants. Experimental intoxication. Monocrotaline. Sheep.

Resumo

Objetivou-se neste trabalho avaliar as alterações clínicas e laboratoriais, bem como identificar e descrever lesões hepáticas presentes em função dos efeitos tóxicos de Crotalaria spectabilis*. Para tanto, 15 fêmeas ovinas (sem raça*

definida) foram alocadas em três grupos de cinco animais. No período experimental de 28 dias, os grupos foram alimentados com ração contendo sementes de C. spectabilis *trituradas, nas porcentagens de 0,4% (G1), 0,6% (G2) e 1% (G3). As amostras sanguíneas e biópsias hepáticas coletadas para exame histológico seguiram o esquema: T0 (dia anterior à inclusão de sementes), T1 (sete dias após a inclusão), T2 (14 dias), T3 (21 dias) e T4 (28 dias). Os resultados foram submetidos à análise de variância (Anova), com auxílio do programa* Statistical Analysis System *(SAS) e tiveram os contrastes de média comparados pelo teste de Tukey (p < 0,05). Apenas um animal (G3 no 21° dia (T3)) apresentou sinais clínicos de intoxicação por* C. spectabillis*. Os parâmetros hematológicos mantiveram-se na faixa de normalidade para a espécie. As atividades séricas das enzimas aspartato aminotransferase (AST) e creatina fosfoquinase (CK) não apresentaram diferenças significativas entre os grupos, enquanto a gamaglutamiltransferase (GGT) apresentou diferença significativa entre G1 (33,66 ± 25,8 U/L) e G2 (67,3 ± 14,8 U/L) no momento controle (T0). Na ultrassonografia evidenciaram-se alterações hepáticas durante o período experimental, sendo confirmadas pelos achados histológicos. Concluiu-se que a análise isolada do hemograma e das provas de função hepática não tem valor diagnóstico para a intoxicação por* C. spectabillis *em ovinos, necessitando a realização de exames complementares, como ultrassonografia e biópsia hepática percutânea.*

Palavras-chave*: Plantas hepatotóxicas. Intoxicação experimental. Monocrotalina. Ovinos.*

Introduction

Plants from the *Crotalaria* genus are widely used as green fertilizer for the recovery of soils that have been depleted by rotational cropping for crops such as maize. They improve the content of nitrogenous compounds in the soil, which are essential for other crops, and they prevent erosion (JOLY, 1977; LORENZI, 1991). However, *Crotalaria* is considered an impurity when present in animal feed. According to Decree Number 845 (November 8th, 1976) of the Ministry of Agriculture, Brazil, impurities are considered to be the product itself as well as its grains or grain fragments that pass through a sieve with 5 mm pores. Therefore, the seeds of *Crotalaria*, which are smaller than 5 mm, are considered impurities if present in animal feed.

Although widely used as green fertilizer, *Crotalaria* plants contain pyrrolizidine alkaloids (PA) that are toxic to animals and humans (COPPLE et al., 2004; KAY; HEATH, 1969; WANG et al., 2005; YAN; COOPER; HUXTABLE, 1995). Several studies have verified the toxicity of *Crotalaria* species in birds and domestic animals (ALFONSO et al., 1993; BURGUERA et al., 1983; CLARKE; CLARKE, 1967; HATAYDE; SOUZA; SANTANA, 1998; MELO, 2010; NOBRE et al., 2005; SOUZA; HATAYDE; BECHARA, 1997; TOKARNIA; DOBEREINER, 1982;). The main pyrrolizidine alkaloid present in *C. spectabilis* is monocrotaline (MCT), which was first isolated by Neal, Rusoff and Ahmann (1935) and can be found in all parts of the plant (PIERCY; RUSOFF, 1946). More importantly, it was found in the seeds (dry weight) at concentrations up to 3.98% (JOHNSON; MOLYNEUX; MERRILL, 1985; WILLIAMS; MOLYNEUX, 1987). Spectabiline, another pyrrolizidine alkaloid, may also be found in small quantities (BULL; CULVENOR; DICK, 1968).

These highly reactive compounds covalently bind to the DNA of hepatic cells and enzymes (MATTOCKS, 1968). They cause liver damage that ranges from edema and centrilobular necrosis, megalocytosis, karyomegaly, fibrosis, bile duct proliferation, and veno-occlusion to complete loss of liver function (CHEEKE; SHULL, 1985; HANUMEGOWDA et al., 2003; SCHULTZE; EMEIS; ROTH, 1996; WANG et al., 2005). According to Copple et al. (2002), dehydromonocrotaline (MCTP) is the metabolite responsible for MCT toxicity "*in vivo*", inducing toxic effects in the liver and lung and triggering the occurrence of pulmonary hypertension.

In this context, the evaluation of liver function becomes important. The pathogenesis of liver diseases in domestic animals is very complex. Therefore, the diagnosis of these diseases involves the dosage of serum biochemical parameters, including the enzymatic activity of aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT), which reveal and confirm the presence of acute or chronic liver damage (TENNANT, 1997), respectively. Furthermore, creatine phosphokinase (CK) is an enzyme widely used to determine muscle changes in

domestic animals and to sensitively and specifically detect muscle damage (CARDINET, 1997).

The diagnosis of liver disease involves a combination of anamnesis, physical and laboratory examinations, and eventually imaging and histology. Under certain conditions, the enzymatic profile does not meet the needs of the clinician to determine the true nature of the liver disease. Liver biopsies allow for a broader view of the disease by determining the morphology of the lesion (TOSTES; BANDARRA, 2002). Another tool is ultrasonographic evaluation of the hepatic parenchyma, although this is a subjective test (NYLAND et al., 2004).

Techniques to quantify the echogenicity and echo texture of the regions assessed may be used to avoid possible misinterpretations and to minimize the subjectivity of the examinations (LU et al., 1997; MAEDA; UTSU; KIHAILE, 1998). One of these techniques is the grayscale method, which allows a quantitative assessment of echo texture and echogenicity of a previously selected region. It shows the frequency and distribution of echo intensity in the region of interest by measuring the amounts of gray levels. This form of analysis is available in many commercial ultrasound machines. However, its application has been restricted to academia, and standardization of values must be expanded prior to use in clinical practice and in experimental protocols in human and veterinary medicine (MAEDA; UTSU; KIHAILE, 1998). Due to a lack of experimental studies that demonstrate the clinical, pathological and biochemical changes in sheep experimentally intoxicated by *C. spectabilis* seeds, this study aimed to evaluate the clinical and laboratory changes in sheep experimentally intoxicated with *C. spectabilis* seeds and to identify any hepatic lesions through liver biopsies guided by ultrasound.

Material and methods

Animals

Fifteen clinically healthy ewes were used in the experiments. They were under breed, approximately three years old and weighed approximately 44 kg. The animals were housed in individual cages equipped with food-troughs and drinking reservoir, and they were located in a covered area next to the Research Support Laboratory of the Clinic and Surgery Department of FCAV-Unesp – Jaboticabal Campus, SP, Brazil. The ewes were subjected to seven days of acclimation prior to the beginning of the experiment. After the adaptation period, the ewes were subjected to daily physical exams in the morning. Behavior, feed and hay intake, conjunctiva mucous membrane color, heart and respiratory rate, rectal temperature and count of ruminal movements were observed daily. The animals were weighed once a week.

Water and hay of "coast cross" grass was provided *ad libitum***,** while the ration of bran, corn and soybeans was limited to 2% of the body weight, according to the standards of the National Research Council – NRC (2007). The meals were offered twice daily, and the *C. spectabilis* seeds were mixed into the morning meal.

This study was approved by the Ethics and Animal Welfare Committee (CEBEA) of FCAV/Unesp under protocol number 012325-8.

Model of experimental intoxication

The animals were randomly divided into three groups with approximate weights of 44 kg. For each group, proportions of 0.4% G_1 (n = 5), 0.6% G_2 (n = 5) and 1.0% G_3 (n = 5) *C. spectabilis* seeds were crushed and mixed into the feed.

The experimental period lasted 28 days and involved blood samples, ultrasonographic evaluations and liver biopsies performed as follows: T0 –day before the initiation of *C. spectabillis* seed inclusion in the feed (control time), T1–seven days, T2–14 days, T3–21 days, and T4–28 days after *C. spectabillis* seed inclusion in the feed. Once signs of intoxication were observed, it was confirmed by clinical and laboratory exams**.** The animals received treatment based on liver protectors[1] and 5% glycophysiological serum[2], and the administration of *C. spectabilis* seeds in the diet was suspended during treatment.

Laboratory tests

Blood samples were collected weekly by jugular venipuncture into sterilized and siliconized vacuum

[1] Mercepton injectable – Bravet Laboratories

[2] Glicophysiological serum 5% - Glicolabor Pharmaceutocal Industry.

tubes[3] with and without the anticoagulant ethylenediaminetetraacetic acid (EDTA) to obtain blood and serum, respectively.

Full blood counts (leukocyte count, red blood cells and hematimetric parameters), liver function evaluations (AST serum activity by the Reitman-Frankel method), GGT, (Szas modified method) and CK (Szas method) were performed.

Ultrasonographic exams and liver biopsies

The ultrasonographic evaluations and subsequent liver biopsies were performed between the 8th and 11th intercostal spaces of the right antimere. In all the animals, the caudal-cranial and dorsal-ventral directions were scanned, evaluating the echogenicity of pattern liver parenchyma. This was considered normal when the liver appeared inside the rib cage with smooth contours and sharp angle margins and with a homogeneous echo texture that was slightly coarser than that of splenic parenchyma. Furthermore, the echogenicity had to be equal to or slightly larger than the renal cortex and smaller than the spleen and the falciform ligament (MAMPRIM, 2004). With the bidimensional images (2D) stored in the computer and with the assistance of specific software, the region of interest (RI) was established to quantify the local average number of pixels in the grayscale and thus to determine the level of echogenicity of the liver. Each RI was bounded by a 10x5x5 mm rectangle located in the central region of the central axis of the resonant bunch at 1 to 2 cm depth.

Liver fragments were collected using the percutaneous biopsy technique with a cutting needle type Tru-Cut[4]. With the animals in quadrupedal position, an area of 10 x 15 cm was shaved off in the right rib cage region (MEDEIROS et al., 2002). For local infiltrative anesthesia, 2 mL of 2.0% lidocaine chlorhydrate without a vasoconstrictor was used (NAVARRE; PUGH, 2005). For this procedure, a 40 x 16 gauge needle was used to pierce the skin and subcutaneous tissues. Then, the cutting needle type Tru-Cut 14G was introduced and drilled into the intercostal muscles up to the liver. The needle was driven in the direction of the opposite elbow joint, forming an angle of 90° to the skin. With the assistance of ultrasound, the necessary depth of penetration of the needle into the liver parenchyma was determined, which measured approximately 2 cm in length and 3 mm wide (FERREIRA; VAN DER MERWE; SLIPPERS, 1996; MEDEIROS et al., 2002; NAVARRE; PUGH, 2005). The liver fragments were placed into flasks containing 10% neutral buffered formalin at pH 7.0 for histological examination.

The liver samples were fixed for more than 18 hours in a liquid:material ratio of 10:1, permitting complete fixation. They were subsequently processed by conventional histological processing with dehydration in increasing dilutions of alcohols and diaphanization in xylene for inclusion into paraffin.

The sections were made in a microtome with 3 µm thickness and were stained by routine hematoxylin and eosin (HE) staining according to the technique described by Luna (1968) for further analysis by light microscopy.

Statistical analysis

Quantitative variables were subjected to analysis of variance (Anova) with the assistance of the Statistical Analysis System (SAS-Version 9.1). The average contrasts were performed by the Tukey posttest with 95% confidence ($p < 0.05$).

Results and discussion

There were no natural deaths of animals during the experimental period. Only one animal in the 1.0% group (G3) began to show signs of intoxication on the 21st day after inclusion (T3), including depression, decreased appetite and ruminal hypomotility. On the next day (the 22nd day), this animal was lying down (sternal recumbency), depressed, lacking appetite, had ruminal hypomotility and ruminal bloat, had smooth feces and was rough haired. The addition of *C. spectabilis* seeds in the feed was suspended, and treatment with 5% glycophysiological serum and liver protectors for three days was realized. This resulted in a prompt response by the animal, which regained its standing position and returned to feed normally.

According to Craig et al. (1991), the different responses or individual resistance of the animals

[3] Vacutainer®, BD Diagnostics - Preanalytical Systems.
[4] Cutting needle type Tru-Cut 14G®

to PA may be a reflection of: 1) bacterial detoxification in the gastrointestinal tract; 2) conversion rate of PA to toxic pyrrole in the liver and; or 3) individual antioxidative activities of the animals. The remaining animals showed no clinical signs of intoxication, and the feed consumptions in different proportions of *C. spectabilis* seeds were complete throughout the experimental period. These results are in agreement with reports by Anjos et al. (2010) in a study with *C. retusa*, in which sheep ate 136.8 mg MCT/kg body weight daily for 20 days and showed no clinical signs. According to Radostits et al. (2002), this seems to result from the sheep's ability to detoxify the PA in the liver, which is likely related to the diet consumed prior to domestication. Small ruminants are selective for food intake and, as a result, have developed resistance to toxins. In this study, the dry matter intake was followed during the experimental period, and no decreases were observed except for one animal from the G3 group that developed clinical signs of intoxication, as previously mentioned.

The animals submitted to the trial did not show marked changes upon the physical examinations. With regards to the body weight of the animals over the 28 days, we observed a significant increase ($p < 0.05$) in the animals of group G2 at all the times evaluated compared with the control time. Likewise, there was weight gain ($p < 0.05$) in the animals of group G3 on the 21st and 28th days, as shown in Table 1. These results differ from the findings of Nobre et al. (2004) for intoxication of horses by *C. retusa* and in Souza, Hatayde and Bechara (1997) for pigs experimentally intoxicated with *C. spectabillis* seeds.

Sheep are likely more resistant due to a greater number of microorganisms in the rumen that can biotransform these alkaloids more efficiently than those in cattle (CRAIG et al., 1991).

The number of leukocytes did not differ ($p < 0.05$) between groups or over time during the experimental period, staying within the normal range quoted by Pugh (2005) for sheep. As for the total neutrophil counts, no changes were observed as per the reference limits established by Pugh (2005). The total erythrocyte counts remained within the physiologic range for sheep. Only within the group with the higher proportion of seeds, G3, was there a below average count on the last experimental day (T4). This result was most likely because one of the sheep in this period had a full blood count below normal ($7.36 \times 10^6/\mu L$), compatible with anemia. In relation to the average values of globular volume throughout the experimental period, no animals showed changes, and there was no significant difference between them. As for the hemoglobin content, no significant differences were found between groups or between time points. A below normal value was only found in group G3 on the 28th day (T4), most likely because one of the sheep had presented a clinical profile compatible with anemia from the second week of treatment.

Regarding the serum activity of gamma-glutamyltransferase (GGT), a decline in the serum concentration of this enzyme was observed after 7 days of seed consumption (T1) in the group with the lowest proportion of seeds in the feed (G1) (59.6 ± 8.9). However, the values remained within the normal range throughout the experimental period, likely because the liver damage observed was not sufficient to induce increased serum activity of GGT, a situation that indicates liver damage and bladder dysfunction according to Tennant (1997).

Table 1 - Means and standard deviations of body weight (kg) of sheep based on period of inclusion of different concentrations of *Crotalaria spectabilis* seeds in feed

Group	T0	T1	T2	T3	T4
G1	49.66 ± 5.72 ^{aA}	50.14 ± 6.45 ^{aA}	50.02 ± 6.39 ^{aA}	50.84 ± 6.44 ^{aA}	50.92±6.43 ^{aA}
G2	42.46 ± 7.11 ^{abA}	45.92 ± 5.76 ^{aB}	46.28 ± 6.20 ^{abB}	47.14 ± 6.26 ^{abB}	47.20±6.41 ^{abB}
G3	34.88 ± 6.22 ^{bA}	35.40 ± 4.99 ^{bA}	36.50 ± 4.42 ^{bAB}	37.28 ± 5.24 ^{bB}	38.14±5.02 ^{bB}

Source: Research data.
Note: Values followed by the same lowercase and uppercase letters in the same column in the same row do not differ by Tukey posttest ($p < 0.05$).

Regarding the serum activity of aspartate aminotransferase (AST), no significant differences were observed between the experimental groups, and the averages were within the normal range quoted by Kramer and Hoffmann (1997). Likewise, the serum activity of creatine phosphokinase (CK) did not change ($p < 0.05$) between the groups or experimental times.

The ultrasonographic evaluations were performed as per Néspoli et al. (2009) in the right antimere of the rib cage between the 8th and 11th intercostal spaces, demonstrating that, in sheep, the liver can be scanned from the 8th to the 12th intercostal space. This evaluation revealed that the liver increased in size in all the animals over time, and the points used for the liver biopsies became increasingly caudal. Ultrasonography revealed a homogeneous echo texture, changes in the architecture of the liver parenchyma, and vascular and echogenicity changes at the control time (T0), consistent with Biller, Kantrowitz and Miyabayashi (1992).

Similarly, a change in the structure of the liver parenchyma in all the animals was observed beginning 7 days after the inclusion of seeds in the feed (T1) and becoming more severe over time. This change was more marked in the group with the higher percentage of seeds (G3). The liver receives products and toxins from the gastrointestinal tract by portal blood flow. Therefore, primary intestinal diseases that damage the mucosa may cause increased absorption of these substances into the portal circulation. These substances can cause liver damage (toxic hepatopathy), such as that caused in this experiment, or they can induce immune responses that lead to the development of inflammation (CULLEN; MacLACHLAN, 2001). The visualization of these changes was possible by the alterations in the echogenicity of the images, which proved increasingly hypoechogenic with increased liver congestion. A hypoechoic liver is associated with diseases that lead to fluid accumulation in hepatocytes, such as acute hepatitis, cholangiohepatitis, passive congestion, and liver necrosis (BILLER; KANTROWITZ; MIYABAYASHI, 1992; BOROFFKA, 1998). Furthermore, it was possible to monitor the dilation of the hepatic portal vein (Figure 1).

Regarding the number of pixels (Table 2 and Graph 1), we found a significant and progressive decrease in the three groups during all the experimental periods. The lowest number of pixels was observed in the group with the lowest percentage of seeds in the feed (G1). This finding reflects an increase in liver congestion. According to the literature (BILLER; KANTROWITZ; MIYABAYASHI, 1992), a diffuse decrease of echogenicity may be related to inflammatory or congestive processes, consistent with the findings of this study.

The histological findings are shown in Figures 3 through 5. The ewes of group 1 did not show histological changes at the control time (T0), the 7th (T1) or 14th (T2) day of seed consumption. However, on the 21st (T3) and 28th (T4) days after the inclusion

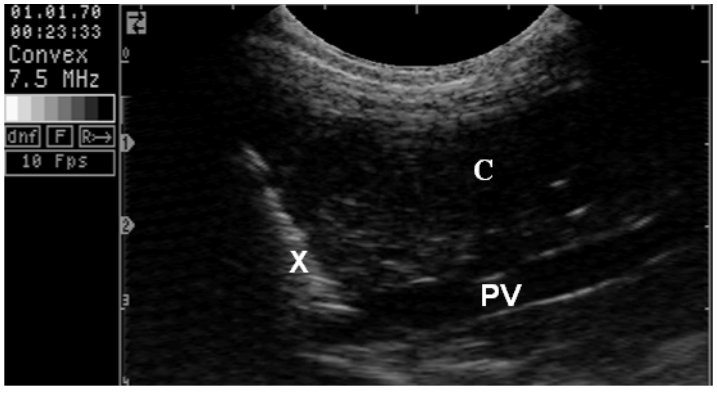


Figure 1 - Ultrasonography in the transverse plane of the liver parenchyma of a G3 ewe on the 14th day after the inclusion of seeds (T2), showing the dilatation of portal vein (PV), liver congestion (C), and hyperechogenicity due to the echo reverb effect at trigger time (X)

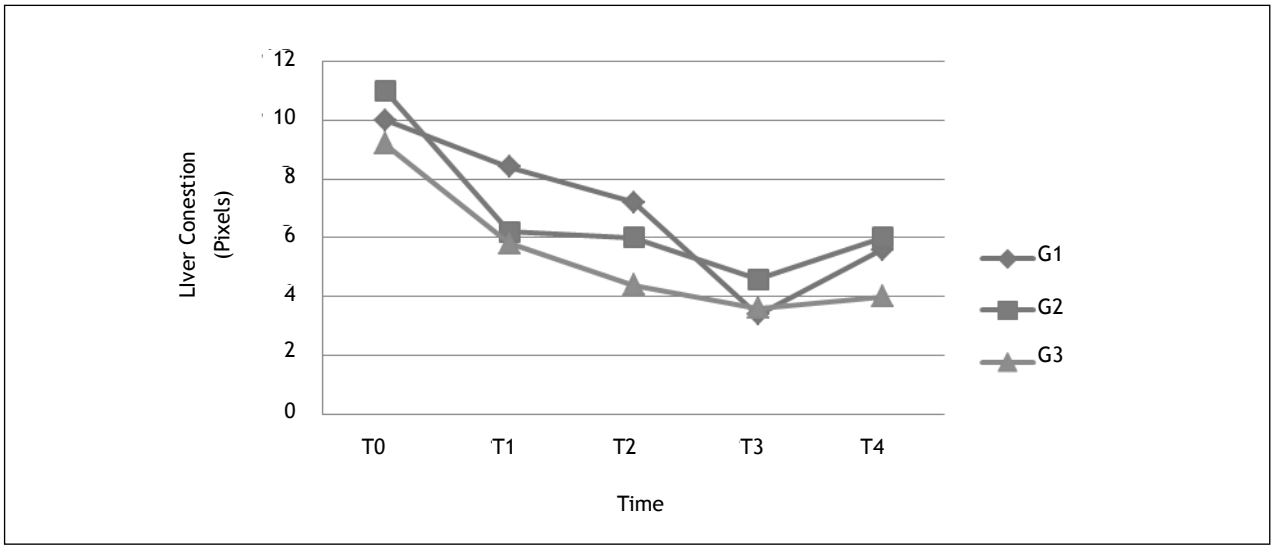
Source: Research data.

Table 2 - Averages and standard deviations of the number of pixels according to the time of inclusion of different concentrations of *Crotalaria spectabilis* seeds in the feed, Unesp – Jaboticabal, 2010

Group	T0	T1	T2	T3	T4
G1	10.0 ± 7.0^{aAC}	8.4 ± 11.2^{aA}	7.2 ± 5.2^{aAC}	3.4 ± 2.8^{aBC}	5.6 ± 6.4^{aC}
G2	11.0 ± 6.0^{aA}	6.2 ± 5.0^{bAB}	6.0 ± 3.8^{aAB}	4.6 ± 3.8^{aB}	6.0 ± 3.8^{aB}
G3	9.2 ± 8.0^{aA}	5.8 ± 7.0^{bAB}	4.4 ± 2.8^{aAB}	3.6 ± 2.6^{aB}	4.0 ± 2.6^{aB}

Source: Research data.

Note: Values followed by the same small letter in the same column and capital letters in the same line do not differ by the Tukey posttest ($p < 0.05$).



Graph 1 - Representation of the number of pixels according to the time of inclusion of different concentrations of Crotalaria spectabilis seeds in the feed

Source: Research data.

of seeds, the animals showed distinct liver changes, such as diffuse hydropic degeneration, multifocal infiltration of polymorphonuclear cells, diffuse fatty degeneration, and multifocal and centrilobular infiltration of mononuclear cells.

In the group with 0.6% seeds (G2), a distinct diffuse hydropic degeneration, multifocal inflammatory infiltration of polymorphonuclear cells and multifocal diffuse fatty degeneration were evident. In one G2 animal at T1, accentuated periportal infiltration of polymorphonuclear cells, bile duct proliferation with fibrosis and moderate multifocal necrosis were observed. These findings are compatible with those described by Dantas et al. (2004) in spontaneous chronic intoxication by *C. retusa* in sheep. He also noted periportal fibrosis and bile duct proliferation. Lemos and Barros (1998) in cattle, Nobre et al. (2004) in intoxication by *C. retusa* in horses and Nobre et al. (2005) in intoxication by *C. retusa* in sheep reported that there were various patterns of degeneration and necrosis of hepatocytes.

On 14th day of seed consumption (T2) moderate inflammatory infiltration of polymorphonuclear cells, fatty degeneration, large areas of moderate multifocal necrosis with multifocal and diffuse inflammatory infiltration of polymorphous and mononuclear cells (Figure 2) was observed in one animal in the G2 group. This finding is consistent with reports by Ilha et al. (2001) of an outbreak of spontaneous intoxication by *Senecio brasiliensis* in sheep, which included variable degrees of hepatocellular degeneration characterized by the accumulation of numerous lipid drops in the hepatocyte cytoplasm

and the presence of lysosomes loaded with electro dense material, which in most cases, correspond to lipofuscin-ceroid. The genus *Senecio* (Asteraceae) belongs to the group of plants containing hepatotoxic pyrrolizidine alkaloids.

A ewe in the group with the greater inclusion of seeds (G3) demonstrated accentuated periportal inflammatory infiltration of mononuclear cells and moderate multifocal and distinct multifocal necrosis at T2 (Figure 3), differing from the findings of Nobre et al. (2005), who reported histological lesions in the liver characterized by centrilobular necrosis. The centrilobular necrosis occurs because there is a higher concentration of cytochrome p450 that metabolizes substances that reach the liver. When the substance is very toxic, it damages the portal space through which it arrives. It is possible that the dose was too high and damaged the adjacent hepatocytes to the portal space, causing the inflammatory infiltrate. Perhaps only one animal was affected because it was more sensitive.

All the animals of the experimental groups, except those from the control time, had inflammatory infiltration of polymorphonuclear or mononuclear cells and hydropic degeneration (Figure 4), thereby characterizing the inflammatory process established in the liver parenchyma.

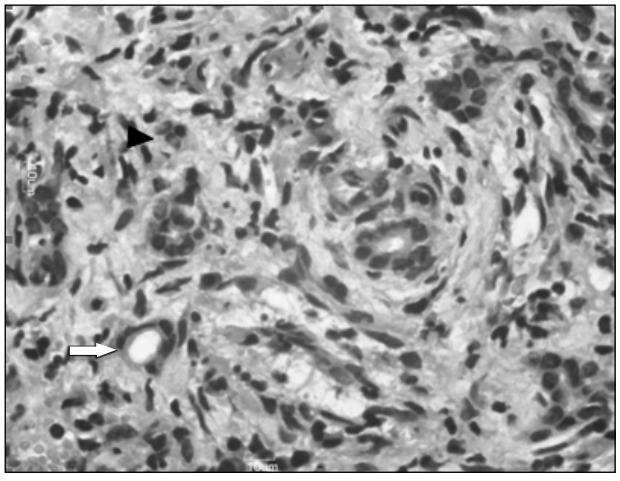


Figure 2 - Liver photomicrograph of a G2 ewe on the 7th day of intoxication (T1), illustrating the presence of accentuated periportal infiltration of polymorphonuclear cells (black arrowhead) and bile duct proliferation with fibrosis (black arrow)

Source: Research data.

Note: H&E staining, 400X magnification.

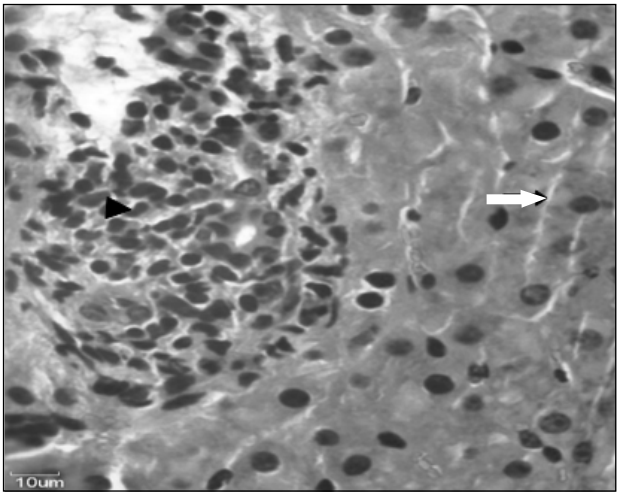


Figure 3 - Photomicrograph of a G3 ewe on the 14th day of intoxication (T2), illustrating the presence of accentuated inflammatory infiltration of mononuclear cells (arrowhead) and distinct multifocal infiltration of polymorphonuclear cells (black arrow). Necrotic area (white arrow)

Source: Research data.

Note: H&E staining, 400X magnification.

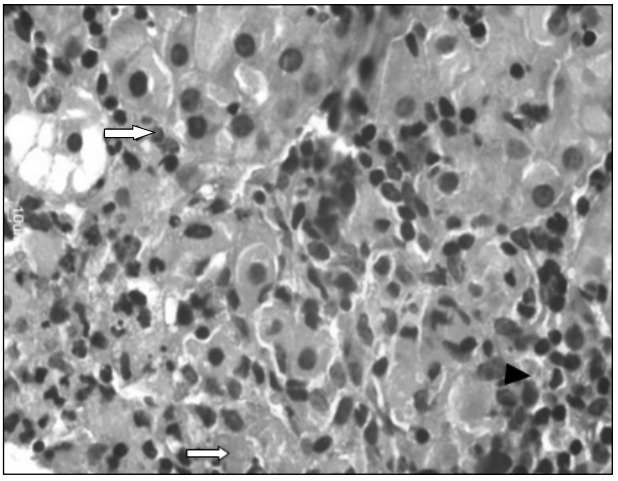


Figure 4 - Photomicrograph of a G3 ewe on the 14th day of intoxication (T2), illustrating the presence of accentuated periportal inflammatory infiltration of mononuclear cells (arrowhead) and hydropic degeneration (black arrow)

Source: Research data.

Note: H&E staining, 400X magnification.

Nobre et al. (2004), Nobre et al. (2005), Boghossian et al. (2007) and Souza, Hatayde and Bechara (1997) cited megalocytosis of hepatocytes as a

common finding during intoxication by pyrrolizidine alkaloids. These authors reported large cytoplasms and nuclei and condensed chromatin at the periphery of the nucleus, a finding that was not observed in this study, suggesting that the different doses and times of exposure to the toxin were not enough to cause such changes.

Conclusions

Ewes experimentally intoxicated with *C. spectabillis* seeds when crushed and incorporated into the feed at rates of 0.2% and 0.4% did not develop clinical signs of toxicity.

A 0.6% level of *C. spectabillis* seeds in the feed may lead to clinical signs of toxicity in sheep individually predisposed to intoxication.

Full blood count and liver function tests have no diagnostic value for intoxication by *C. spectabillis* seeds in sheep, making it necessary to perform other examinations, such as ultrasonography and percutaneous liver biopsy.

The lesions observed in the histological evaluations of sheep liver fragments subjected to continuous ingestion of *Crotalaria spectabilis* seeds at rates of 0.2%, 0.4% and 0.6% were compatible with hepatotoxicity. However, they were not sufficient to alter the serum activities of gamma-glutamyltransferase, aspartate aminotransferase and creatine phosphokinase.

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