

CLINICAL AND PATHOLOGICAL CHANGES OF LAYING HENS (*Gallus gallus domesticus*) EXPERIMENTALLY POISONED BY *Crotalaria spectabilis* SEEDS (Leg. Papilionoideae)

ALTERAÇÕES CLÍNICO-PATOLÓGICAS DE GALINHAS POEDEIRAS
(*Gallus gallus domesticus*) INTOXICADAS EXPERIMENTALMENTE
COM SEMENTES DE *Crotalaria spectabilis* (Leg. Papilionoideae)

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SUMMARY

C. spectabilis belongs to *Crotalaria* genus of the *Leguminosae* family, and is used as “green manuring”. In that manner, its seeds may be harvested along with corn and soy beans contaminating animal foods. The purpose of this work was to evaluate the toxicity of *C. spectabilis* seeds present in the diet fed to laying hens. Eighty “Hisex White” fowls were divided into four groups G₁, G₂, G₃ and G₄. The laying hens were fed diets containing 0.00, 0.02, 0.04 and 0.06% of crushed seeds of *C. spectabilis* for 28 days. Every day, fowls were monitored clinically. The fowls that died in the course of the experiment and the fowls euthanized at the end, were necropsied and fragments of liver, kidney, proventricle, lungs and heart were collected for histopathology. Macroscopic examination of poisoned fowls revealed ascites, cachexia, liver volume increased or reduced with fibrin or subcapsule hematomas. Histopathology showed steatosis, congestion, hemorrhage, megalocytosis and necrosis of hepatocytes. Therefore, the *C. spectabilis* seeds incorporated into diets at levels of 0.02%, 0.04% and 0.06% were hepato-toxic for laying hens.

KEY-WORDS: Hepatic changes. Monocrotaline. Pyrrolizidine alkaloids

RESUMO

A *C. spectabilis* pertence ao gênero *Crotalaria*, família *Leguminosae* e é utilizada como adubação verde. Desta forma, suas sementes podem ser colhidas juntamente com os grãos de milho e soja contaminando alimentos para animais. Para avaliar a toxicidade das sementes de *C. spectabilis* para galinhas poedeiras, foram utilizadas 80 galinhas “Hisex White”, distribuídas nos grupos G₁, G₂, G₃ e G₄, que receberam durante 28 dias, rações contendo respectivamente: 0,00, 0,02, 0,04 e 0,06% de sementes trituradas de *C. spectabilis*. As aves que morreram durante o experimento, e as, sacrificadas ao final do mesmo foram necropsiadas colhendo-se fragmentos de fígado, rim, pró-ventrículo, pulmão e coração para histopatologia. Macroscopicamente verificou-se ascite, caquexia, fígado reduzido ou aumentado de volume com presença de fibrina e/ou hematomas subcapsulares. Microscopicamente, encontrou-se esteatose, congestão, hemorragia, megalocitose e necrose de hepatócitos. Assim, conclui-se que as sementes de *C. spectabilis* incorporadas na ração aos níveis de 0,02%, 0,04% e 0,06% são hepatotóxicas para galinhas poedeiras.

PALAVRAS-CHAVE: Alcalóides pirrolizidínicos. Alterações hepáticas. Monocrotalina

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INTRODUCTION

The *Crotalaria spectabilis* belongs to the Leguminosae family, Papilionoideae subfamily and is distributed mainly in tropical and subtropical areas of the planet (WILLIAMS & MOLLYNEAUX, 1987) and it is mainly used as “green manure” in corn and soybean plantations. For this purpose, *C. spectabilis* is planted and subsequently incorporated into the soil in order to preserve and/or restore soil nutrients and organic matter (SILVA et al., 1999). However, after incorporation into the soil, crotalaria may grow spontaneously in these plantations, and its seeds are harvested together with corn or soybean contaminating them.

Once ingested, these seeds may poison both animals and humans, since they contain the pyrrolizidine alkaloid, monocrotaline, which in addition to toxic effect has also tumorigenic activity (FU et al, 2002, COPPLE et al., 2004, WANG et al., 2005). Natural cases of poisoning have been reported in horses, cattle, sheep, goats, pigs, poultry and humans (FU et al., 2002, NOBRE et al., 2004).

The monocrotaline in the liver causes necrosis of centrilobular parenchymal cells, damage to endothelial cells of the central venules and sinusoids, resulting in congestion and dilation of sinusoids, in addition to hemorrhage and activation of coagulation with subsequent deposition of fibrin in the centrilobular regions (COPPLE et al., 2004). In the early acute phase, the endothelial cells of sinusoids and hepatic venules, as well as liver parenchymal cells are damaged. Later, the subacute phase that follows, consists of the obliteration of intrahepatic venules associated with fibrosis of the sinusoids, a process called: veno-occlusive disease or sinusoidal obstruction syndrome (COOPPLE et al., 2003). Chronic exposure to this substance also causes megalocytosis, steatosis (fatty degeneration), enlarged nuclei with higher density of nuclear chromatin, loss of metabolic function, mitosis inhibition, proliferation of bile ducts, liver cirrhosis, nodular hyperplasia and adenomas or adenocarcinomas (WANG et al., 2005).

Several reports in the literature investigate the toxicity of crotalaria species to birds and domestic animals (ALFONSO et al., 1993, HATAYDE et al., 1997a, HATAYDE et al., 1997b, SOUZA et al., 1997, NOBRE, et al., 2004). However, there are no reports about poisoning of laying hens with seeds of *C. spectabilis*. Thus, the objective of this work was to evaluate poisonous effects of *Crotalaria spectabilis* seeds, crushed and added to the diet of laying hens by observing possible clinical and pathological changes resulting from the poisoning.

MATERIAL AND METHODS

Eighty healthy “Hisex White” laying hens, which were donated to the Poultry Sector of the Faculdade de Ciências Agrárias e Veterinárias, FCAV-UNESP, Jaboticabal, were used. The hens, aged 72 weeks at the beginning of the experiment, were distributed randomly in four groups of 20 hens each (G₁, G₂, G₃

and G₄), kept in individual laying cages, equipped with nipple type water drinker and collective wooden feeders that could feed up to 10 hens, and separated by a distance of 1.60 m. The hens were fed 110 g/hen/day of a laying maintenance ration, formulated according to recommendations from the National Research Council – NRC, where the main source of carbohydrate and protein were respectively, corn and soy, with no coccidiostats drugs added. The ration was manufactured with selected ingredients by the FCAV-UNESP in Jaboticabal. Water was given *ad libitum*. Experimental groups were divided according to inclusion levels of previously crushed *C. spectabilis* seeds added to the ration:

G₁ – Maintenance ration (without seeds) – Control Group;

G₂ – Maintenance ration + 0.02% seeds (20.0 g seeds/ 100.00 kg ration)

G₃ – Maintenance ration + 0.04% seeds (40.0 g seeds/ 100,00 kg ration)

G₄ – Maintenance ration + 0.06% seeds (60,00 g seeds/ 100,00 kg ration)

The amount of seeds added to the diet to characterize poisoning levels followed recommendation by Hatayde et al. (1997a).

Daily inspection was held at a distance from the hens, to evaluate behavior, posture and attitude, comparing the two groups, control and poisoned. The hens that displayed behavioral changes were removed from the cage and submitted to physical examination.

The fowls that died during the course of the experiment and the ones euthanized at the end of the 28 days, were necropsied in the Departamento de Patologia Veterinária da FCAV, UNESP, in Jaboticabal. Samples collected from the heart, lung, liver, kidney and proventricle, were fixated in formalin 10% neutral buffered with phosphate during a period of 24 hours. After fixation, the tissues were hardened in paraffin. The tissues were then cut in the microtome into 3.0-micron thick sections that were subsequently stained with hematoxylin and eosin (LUNA, 1968). Sections were examined by optical microscopy.

RESULTS

The main clinical signs observed in the hens of groups G₂, G₃ and G₄ compared to G₁ are described in Table 1.

Clinical signs of poisoning became evident from the second week on in the hens of G₄, and three died during the period. The hens also became anorexic, lost weight, presented dyspnea, bulging abdomen, ruffled feathers and pale ridges. In the third week, the hens from G₃ began to show milder clinical signs of poisoning compared to hens from G₄, bulging abdomen, anorexia, depression and pale mucous membranes; and at the end of this week, three hens died. In the last seven days of the experiment, hens from G₂ showed decreased feed intake, weight loss and bulging abdomen. Clinical signs were similar among the hens of poisoned groups; however, the hens from G₄ displayed more severe signs. During the 28 days of the experiment, a total of 14 hens died, eight from G₄,

five from G₃ and one from G₂. In G₁ there was no mortality.

The fowls that died during the course of the experiment and the ones euthanized in the last day, were necropsied. Macroscopic lesions were found in hens of groups G₂, G₃ and G₄. Similar changes with different intensity were observed in the groups, as shown in Table 2.

Cachexia was found due to deformation of the keel and atrophy of the breast muscle of the poisoned hens, and this finding was most evident in hens from G₄. Another frequent finding in hens of groups G₂, G₃ and G₄ was the presence of ascites fluid, yellowish, reddish or hemorrhagic (Figure 1A). Often, there were fibrin deposits on the viscera (Figure 1B) and/or adhered to the liver capsule (Figure 1B-arrow). The most severe lesions were observed in the livers of the hens that showed congestion, hemorrhage and sub-capsular hematoma (Figure 1C) resulting from the rupture points of the hepatic parenchyma, causing blood to stay logged between the liver capsule and the parenchyma, without overflowing into the cavity. Other changes observed were: reduced size, yellow-colored liver, firm in consistency with irregular surface and spots of fibrin deposition on the capsule, as well as yellow nodules randomly distributed throughout the parenchyma (Figure 1D). Other organs did not show macroscopic lesions.

Microscopic examination of the organs of G₁ hens, showed that three birds presented mild steatosis.

Several lesions were observed in the livers of hens from groups G₂, G₃ and G₄. With different levels of severity, it was found: fatty degeneration and necrosis of hepatocytes (Figures 2A and 2B), megalocytosis of hepatocytes (Figures 2A and 2C), hemorrhage (Figure 2D), proliferation of fibrous connective tissue (Figures 2A and 2C) and bile ducts (Figures 2B and 2C). The bleeding was also extensive, forming large clumps of blood (hematoma) or infiltrating among hepatocytes (Figure 2D). Sinusoidal congestion, capsular fibrosis, intrahepatic cholestasis and a mixed inflammatory infiltrate were also seen, but less frequently.

Due to recurrent liver histopathological lesions observed in all hens, intensity scores were assigned and classified as shown in Table 3. For this classification 10, 11, 12 and 14 blades were chosen randomly from groups G₁, G₂, G₃ and G₄, respectively. Thus the scores were as follows: 1- mild lesions, degeneration and megalocytosis; 2- degeneration, megalocytosis, marked proliferation of connective tissue and bile ducts; 3- degeneration, megalocytosis, marked proliferation of connective tissue, mild hemorrhage, necrosis of small areas; and, 4- degeneration, megalocytosis, extensive necrosis, profuse hemorrhage, intensive proliferation of connective tissue and bile ducts.

In the kidneys, degeneration and necrosis of cortical renal tubules were observed in 2, 3, 5 and 7 hens of groups G₁, G₂, G₃ and G₄, respectively. The heart, lungs and proventricle of hens in groups G₂, G₃ and G₄ did not show any histopathological changes.

Table 1 - Main clinical signs observed in hens experimentally poisoned (0.02%, 0.04% and 0.06%) by *Crotalaria spectabilis* seeds compared to the control group (0.00%)

Clinical signs	Experimental groups			
	G1 (0.00%)	G2 (0.02%)	G3 (0.04%)	G4 (0.06%)
Anorexia	0/ 20 ¹	5/ 20	12/ 20	20/ 20
Weakness	0/ 20	5/ 20	12/ 20	19/ 20
Dyspnea	1/ 20	0/ 20	10/ 20	20/ 20
Ruffled feathers	0/ 20	6/ 20	11/ 20	18/ 20
Depression	0/ 20	4/ 20	9/ 20	19/ 20
Pale mucous membranes	0/ 20	3/ 20	4/ 20	10/ 20
Bulging abdomen	0/ 20	10/ 20	18/ 20	20/ 20
Weight loss	0/ 20	5/ 20	20/ 20	20/ 20
Non-coordination	0/ 20	0/ 20	2/ 20	3/ 20

Note: ¹ number of affected hens/ total hens in the group

Table 2 - Main findings resulting from the necropsy of hens experimentally poisoned (0.02%, 0.04% and 0.06%) by seeds of *Crotalaria spectabilis* compared to control group (0.0%)

Necropsy findings	Group			
	G1	G2	G3	G4
Cachexia	0/ 20	3/ 20	10/ 20	20/ 20
Pale mucous membranes/ ridge	0/ 20	10/ 20	11/ 20	13/ 20
Ascites	0/ 20	15/ 20	20/ 20	20/ 20
Hepatic parenchyma				
• Increased	2/ 20	6/ 20	3/ 20	0/ 20
• Decreased	0/ 20	3/ 20	12/ 20	18/ 20
• Deposition of fibrin	0/ 20	10/ 20	16/ 20	17/ 20
• Subcapsular hematoma	0/ 20	8/ 20	11/ 20	15/ 20
• Congestion/ hemorrhage	0/ 20	14/ 20	16/ 20	15/ 20
• Fibrosis	0/ 20	3/ 20	10/ 20	18/ 20
• Necrosis	0/ 20	3/ 20	12/ 20	18/ 20

Table 3 - Number of hens experimentally poisoned (0.02%, 0.04% and 0.06%) by seeds of *Crotalaria spectabilis* compared to control group (0.00%) and scores of liver lesions intensity .

Intensity of liver lesions (scores)	Group			
	G1	G2	G3	G4
1	0	3	2	0
2	0	4	2	6
3	0	1	4	3
4	0	3	4	5
Total blades evaluated	10	11	12	14

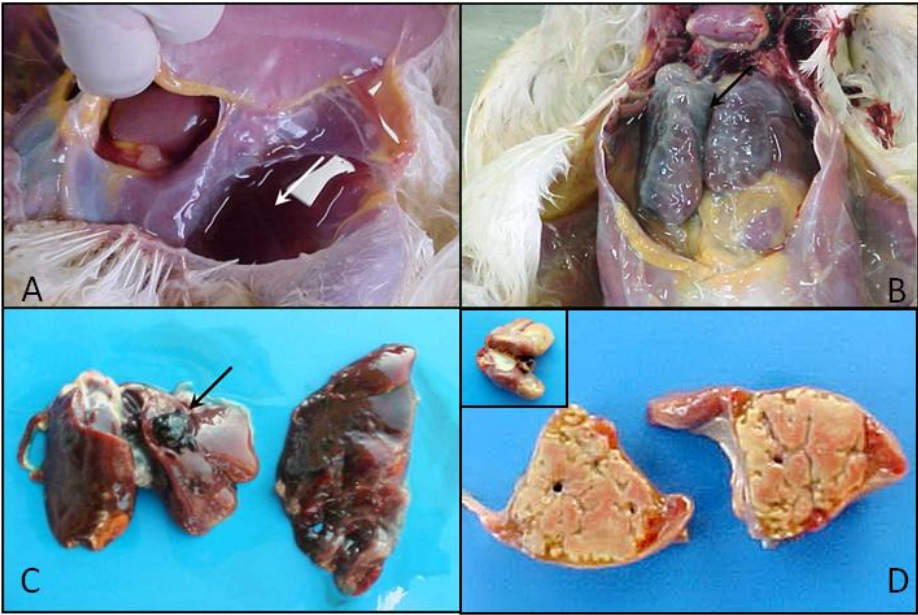


Figure 1 - Experimental poisoning by seeds of *Crotalaria spectabilis* in laying hens. A: Presence of sero-sanguineous fluid in the coelomic cavity (→); B: Presence of fibrin deposits on the liver (→); C: Rupture of hepatic parenchyma with the presence of subcapsular hematoma (→); D: Reduced size, yellow-colored liver, with firm consistency. In detail, it is noticed that the area extends to the cutting surface.

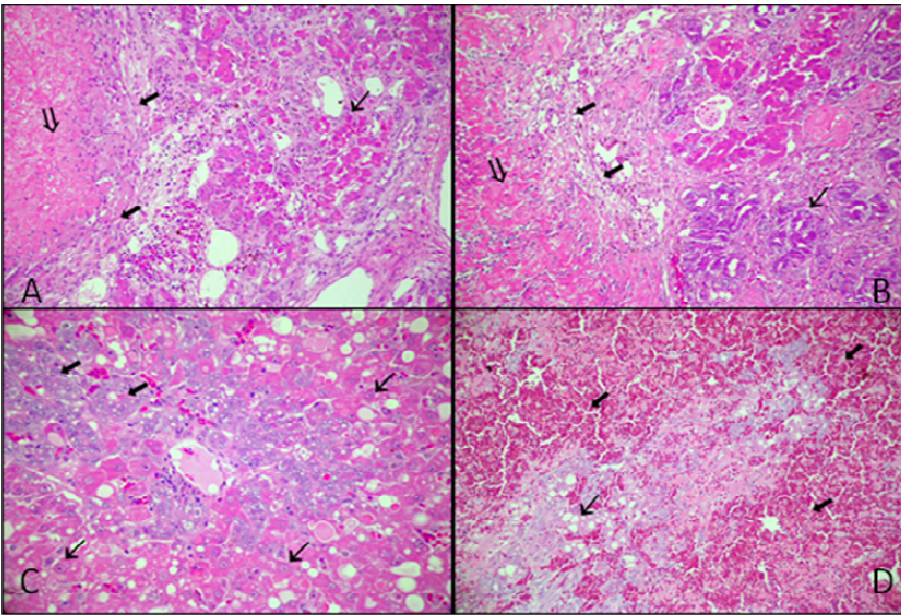


Figure 2 - Experimental poisoning by seeds of *Crotalaria spectabilis* in laying hens. Histopatologia de fígado. A: extensiva área de coagulativa necrosis (⇨), proliferation of fibrous connective tissue (⇨) and megalocytosis of hepatocytes (⇨) (HE, obj. 20X); B: proliferation of bile ducts (⇨), beyond coagulativa necrosis (⇨) and proliferation of fibrous connective tissue (⇨) (HE, obj. 20X); C: proliferation of bile ducts (⇨) and megalocytosis of hepatocytes (⇨) (HE, obj. 40X); D: profuse hemorrhage (⇨) and hepatic steatosis (⇨) (HE, obj. 20X).

DISCUSSION AND CONCLUSION

All hens that ingested the diet containing seeds of *C. spectabilis* showed symptoms of poisoning, which were very similar among groups and differed only in time and intensity of symptom onset, thus showing that the severity of the process is proportional to the dose of poison. The data showed that the levels of seed added to the diet represented a serious problem to the laying hens, and yet the amount of seeds added is small compared to the total amount of ration produced. The ingestion of *C. spectabilis* seeds by groups G₂, G₃ and G₄ caused symptoms very similar to those reported in several studies (ALFONSO et al., 1993, HATAYDE et al., 1997a, HATAYDE et al., 1997b) such as, ruffled feathers, bulging abdomen and dyspnea, except for jaundice, that was not observed in any of the poisoned hens.

The results of the necropsy showed alterations similar to those described by Alfonso et al., (1993), Hatayde et al. (1997a) and Hatayde et al. (1997b), the presence of ascites and reduced size liver, yellowish, with white points and retracted surface covered by fibrin. However, the presence of ascites with sero-sanguineous content or hemorrhage has not been described by these authors. Another characteristic lesion was the presence of rupture points in the hepatic parenchyma with the formation of hematomas located between the parenchyma and liver, as illustrated in Figure 1C. These macroscopic changes were restricted only to the liver, different from what was observed by other authors who have observed macroscopic lesions in kidneys, lungs, and liver as well.

Microscopic examination of the liver showed lesions such as hemorrhage, degenerative changes, hepatocyte necrosis, connective proliferation, steatosis with large and small vacuoles, diffuse or extensive focal infiltrates of the inflammatory cell. Lesions similar to those reported by several authors (ALFONSO et al., 1993, COPPLE et al., 2004, NOBRE et al., 2004). In addition to these lesions, the proliferation of bile ducts, thickening of liver capsule, random bruising and massive necrosis of hepatocytes.

The presence of megalocytosis, the thickening of the nuclear membrane with evident and increased nucleolus was common in poisoned hens. This same lesion was observed in chickens (HATAYDE et al., 1997a), broilers (HATAYDE et al., 1997b), pigs (SOUZA et al., 1997) poisoned with *C. spectabilis* seeds, rats experimentally poisoned with monocrotaline (COPPLE et al., 2004, WANG et al., 2005), humans after ingestion of pyrrolizidine alkaloids contained in dietary supplements (FU et al., 2002) and horses poisoned naturally with *Crotalaria retusa* (NOBRE et al., 2004).

According to Fu et al. (2002), acute poisoning by monocrotaline causes massive hepatotoxicity, hemorrhagic necrosis, while chronic poisoning shows lesions in the lungs and kidneys, in addition to liver. However, this was not observed in this study, since the ingestion of seeds in the proportions of 0.02, 0.04 and 0.06% added to the diet induced chronic cases of poisoning without affecting the lungs or kidneys.

Hepatic changes were remarkable for the hens, and many even showed rupture of the hepatic parenchyma with formation of subcapsular hematomas. Apart from the hepatic rupture, the use of seeds in these proportions can be used to induce experimental ascites in poultry.

Therefore, the ingestion of crushed seeds of *C. spectabilis* added to the diet of laying hens, at the proportions of 0.02, 0.04 and 0.06%, and fed during 28 days caused only hepatic lesions, without damaging lungs and kidneys, and compromised the development and productivity of laying hens as well.

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