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Pathology of cattle experimentally intoxicated with ground *Ricinus communis* seeds

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Abstract

Five Aberdeen Angus calves were inoculated intra-ruminally with ground seeds of *Ricinus communis* at doses of 1, 1.5, 2 or 3 gr per kg of body weight, or with saline solution (control), respectively. Grossly, all intoxicated animals showed hemorrhages in abdominal serosas, epicardium, endocardium, spleen, pre-stomachs, abomasum, and small and large intestine, and diffuse edema of the ruminal mucosa. Microscopically, in all animals inoculated with *R. communis* seeds, the main feature was the presence of pyknotic and karyorrhectic nuclei in the endothelium of central nervous system, hepatic, ruminal, intestinal, glomerular and alveolar capillaries, and in lymphoid cells of multiple organs. Apoptosis, confirmed by activated caspase-3 immunohistochemistry, was observed in these cells. No gross or microscopic lesions were observed in the control animal. The results of this study suggest that apoptosis is the main mechanism of cell death in cattle intoxicated with *R. communis* seeds.

Key words: apoptosis, cattle, experimental intoxication, *Ricinus communis*.

Introduction

Ricinus communis is a plant that belongs to the Eufhorbiaceas family. This plant is toxic because of the presence of two principles: ricinin and ricin. Ricinin is an alkaloid which affects mainly the nervous system, causing convulsions (13), probably due to increased release of glutamate and inhibition of the postsynaptic receptors in the brain (3). Ricin, also called toxoalbumin, is a lectin, which affects mostly the alimentary tract. This toxin is one of the most toxic substances known and is composed of 2 chains of glycoproteins, A and B. Chain A binds galactosidase on the cell surface facilitating endocytosis of ricin, and chain B inhibits protein synthesis, which is the mechanism responsible for the main toxic effect of ricin (3).

Although all parts of the plant are toxic, the seeds of *R. communis* are the most toxic due to a high concentration of ricin. This toxin constitutes between 1 and 5% of the weight of seeds in *R. communis*. Seed chewing by animals is important to break the cuticle and release ricin, and without this step, the seeds may be eliminated intact in feces with not deleterious effect to the animals. Because of this, and the fact that lectins are poorly absorbed in the digestive tract of animals, the incidence of *R. communis* intoxication in animals is low (10). Spontaneous intoxication by this plant has been reported,

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however, in many animal species including dogs, poultry, wild fowl, pigs, horses, sheep, goats and cattle (1, 10).

Clinical signs in ruminants spontaneously and experimentally intoxicated with *R. communis* include weakness, hypersalivation, diarrhea, dehydration, mydriasis, teeth grinding, hypothermia, colic, ruminal stasis and recumbency (1, 2, 13). Gross findings include gastroenteritis, multi-systemic hemorrhages and pulmonary edema, while microscopic lesions are characterized by multi-systemic hemorrhages and necrosis, gastroenteritis and pulmonary edema (1, 2).

Experimental intoxication with seeds of *R. communis* by oral or intra-gastric inoculation has been previously described in cattle (13, 14). Detailed descriptions of the pathology of this intoxication in this species are, however, scant and the mechanism of cell death has not been described in this species. The objective of the present study was to describe in detail the gross and microscopic pathology, with emphasis on the mechanisms of cell death, in cattle experimentally intoxicated with *R. communis* seeds.

Material and methods

Three male and two female healthy Aberdeen Angus calves were used. All animals were between 7 and 18 months of age and weighed between 85 and 217 kg (Table 1). A rumenotomy was performed on all animals. For this, the animals were fasted for 24 hs, but they were offered water ad-libitum. Anesthesia was induced and maintained by intravenous ketamine (Phoenix, St. Joseph, MO) and xylazine (Lloyd, Shenandoah, IA). In addition, an epidural injection of 2% lidocaine (Sparhak, Lenexa, KS) was performed, and a 0.5% solution of this drug was also injected in the area of incision. A laparotomy was performed via the left flank and the rumen was exposed and incised before 4 animals (1, 2, 3, 4) were inoculated intra-ruminally with ground seeds of *R. communis* diluted in normal saline solution at doses of 1, 1.5, 2 and 3 gr per kg of body weight, respectively (Table 1). Only saline solution was introduced in the rumen of the fifth animal which was considered a negative control. The rumen and abdominal wall were closed with separate sutures. The *R. communis* seeds had been collected in Cerrillos, Salta Province, Argentina, they were grounded to obtain particles 2-4 mm diameter and stored for 5 months at room temperature until used for this experimental intoxication.

All animals were clinically evaluated regularly before and after inoculation, including observation of clinical signs, and measurement of heart and respiratory rates, and rectal temperature. The negative control and animal 1 were euthanized 38 and 21 hs after inoculation, respectively (Table 1), while the others 3 inoculated animals died spontaneously at 38, 19 and 21 hs after inoculation, respectively (Table 1). Euthanasia was performed with an overdose of barbiturate. All procedures involving animals were performed following the guidelines of Institutional Committee for Care and Use of Experimental Animals from the Regional Center Buenos Aires Sur of the National Institute of Agricultural Technology (Permit # 134-2017).

Table 1. Signalment, inoculation dose, interval inoculation-death and form of death in four calves intoxicated with seeds of *R. communis* and a control calf.

Animal	Sex	Age (months)	Weight (kg)	Inoculation dose (gr/kg of body weight) of *R. communis* seeds	Interval inoculation death (hs)	Form of death
1	Female	7	85	1	21	Euthanasia
2	Male	18	200	1,5	38	Spontaneous
3	Male	12	217	2	19	Spontaneous
4	Female	7	150	3	21	Spontaneous
5	Male	8	149	0	38	Euthanasia

Necropsy was performed on all animals immediately after death, and samples of rumen, small intestine, colon, spleen, kidney, heart, liver, brain, lung and mesenteric lymph nodes were collected and fixed by immersion in 10% buffered formalin for 24 to 72 hours. All tissues were processed by standard techniques for the production of 4µm thick hematoxylin and eosin (H&E) stained sections.

Sections of liver, mesenteric lymph nodes, spleen, kidney, rumen, small intestine and colon from animals 3 and 4 were also processed by immunohistochemistry (IHC) for activated caspase-3 using the Dako EnVision Kit (Dako, Carpenteria, California) according to the manufacturer's instructions. A rabbit polyclonal anti-cleaved caspase-3 antibody (ASP175, Cell Signaling Technology, Inc, Danvers, MA) was used as the primary reagent at a 1:100 dilution, and the immunoreactivity was visualized with the Nova Red chromogen (Vector laboratories Inc, Burlingame, CA). A lymph node from a cow with lymphoma was employed as a positive control.

Sections of liver from animals 3 and 4 were also processed for IHC for ionized calcium-binding adaptor molecule 1 (Iba1), using a primary rabbit polyclonal antibody against this protein (Wako Pure Chemical Industries, Osaka, Japan) and a streptavidin system (Pierce, Pasadena, CA), according to the instructions of the manufacturer. The reaction was visualized with the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St Louis, MO). In addition, IHC for detection of endothelial cells was performed on sections of liver, kidney, lung, rumen, small intestine and colon from these two animals using a primary rabbit polyclonal antibody against von Willebrand factor VIII (Dako, Carpenteria, California) at a 1:2000 dilution. The avidin–biotin–peroxidase method was employed according to manufacturer's instructions (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA). Positive immunoreactivity was visualized using the chromogen 3-amino-9-ethylcarbazole (AEC K4001, Dako). Endothelial cells in blood vessels of the sections were considered positive controls. Rabbit IgGs were used as primary antibodies in negative control sections for all IHC tests.

Results

Animal 1 showed increased heart rate (130 beats/min) shortly before euthanasia 21 hours after inoculation (Table 1). Animal 3 showed, for a short period before death at 19 hours after inoculation, increased heart rate (130 beats/min), depression, bloody diarrhea, sternal recumbency, slight muscle tremors and incoordination with pendulous movements of the head (Table 1). Animals 2 and 4 showed convulsions soon before death at 38 and 21 hours post-inoculation, respectively (Table 1). No clinical abnormalities were observed in the negative control animal which was euthanized 38 hs after the rumenotomy.

Grossly, all intoxicated animals showed petechial hemorrhages in abdominal serosas, epicardium, endocardium and spleen. In addition, these animals showed diffuse, moderate to severe edema of the ruminal mucosa and sub-mucosa (Fig. 1), and diffuse congestion and hemorrhage of the mucosa of all pre-stomachs, abomasum, and small and large intestine. These lesions were most severe in the colon and cecum (Fig. 2). The content of the small intestine was liquid and dark red, and the content of the colon and cecum was dark red with multiple blood clots. The liver was enlarged and congested, and had rounded edges. The mesenteric lymph nodes were enlarged and edematous. The wall of the gall bladder and of the proximal portion of the duodenum was edematous.

Microscopically, the most striking finding in all intoxicated animals was the presence of pyknotic and karyorrhectic nuclei in the endothelial cells of most capillaries within the brain, liver (Fig. 3), rumen, intestine, renal glomeruli and pulmonary alveoli. In addition, the ruminal mucosa of animal 2 had multifocal coagulation necrosis, diffuse infiltration of viable and degenerated neutrophils, microthrombi in capillaries and arterioles, hemorrhage and edema. The small intestine and colon of animals 2, 3 and 4 had diffuse and transmural congestion and hemorrhage. The Peyer's patches of animal 4 showed severe lymphoid depletion with pyknosis and karyorrhexis of lymphoid cells. The liver of animal 3 had several areas of focally extensive, random, coagulative necrosis surrounded by a rim composed of a few degenerated and viable neutrophils. The liver of animal 4 had severe centrilobular degeneration, disruption of hepatic cords and individual hepatic cell necrosis. The portal areas of animals 3 and 4 had mild infiltration of lymphocytes and macrophages, and cell debris. In addition, the mesenteric lymph nodes of these two animals showed severe, diffuse subcapsular hemorrhage, multifocal hemorrhages in the cortex, dilatation of medullary sinuses and depletion of lymphocytes in the germinal centers with pyknosis and karyorrhexis of lymphoid cells. The spleen of these two animals had lymphoid depletion in the follicles, with severe pyknosis and karyorrhexis of lymphoid cells. The kidneys of all intoxicated animals had a slight to moderate amount of eosinophilic amorphous material in the lumen of the tubules and Bowman's space and multifocal tubular necrosis. The lungs of all intoxicated animals presented mild, focal, intra-alveolar and interlobular edema, with moderate congestion, and thrombi in alveolar capillaries. The heart of animal 4 showed severe diffuse sub-epicardial hemorrhage and individual myocardiocyte necrosis. The severity of both gross and microscopic changes was dose dependent with animal 1 showing mild changes and animal 4 showing the most severe changes. No microscopic lesions were observed in any of the tissues of the negative control animal examined.

IHC for activated caspase 3, Iba I and von Willebrand factor VIII was performed in animals 3 and 4. The immunolabeling against activated caspase 3 revealed positive nuclear immunostaining in the endothelial cells of all tissues which showed pyknosis and karyorrhexis (Fig. 4 and 5). These cells stained positively for von Willebrand factor VIII (Fig. 5). Positivity for activated caspase 3 was also observed in the hepatocytes of the periportal area, Kupffer cells, and lymphoid cell aggregates of the colonic mucosa (Fig. 6) follicles of the mesenteric lymph nodes, Peyer's Patches and spleen. Iba I was used to identify macrophages in all organs examined.

Discussion

The clinical and pathological findings associated with ricin intoxication depend on route, dose of inoculation and susceptibility of the animal species involved (5, 13). Likewise, in this study, the severity of the lesions in multiple organs was dose-dependent. This however, must be interpreted with caution as only a very small number of

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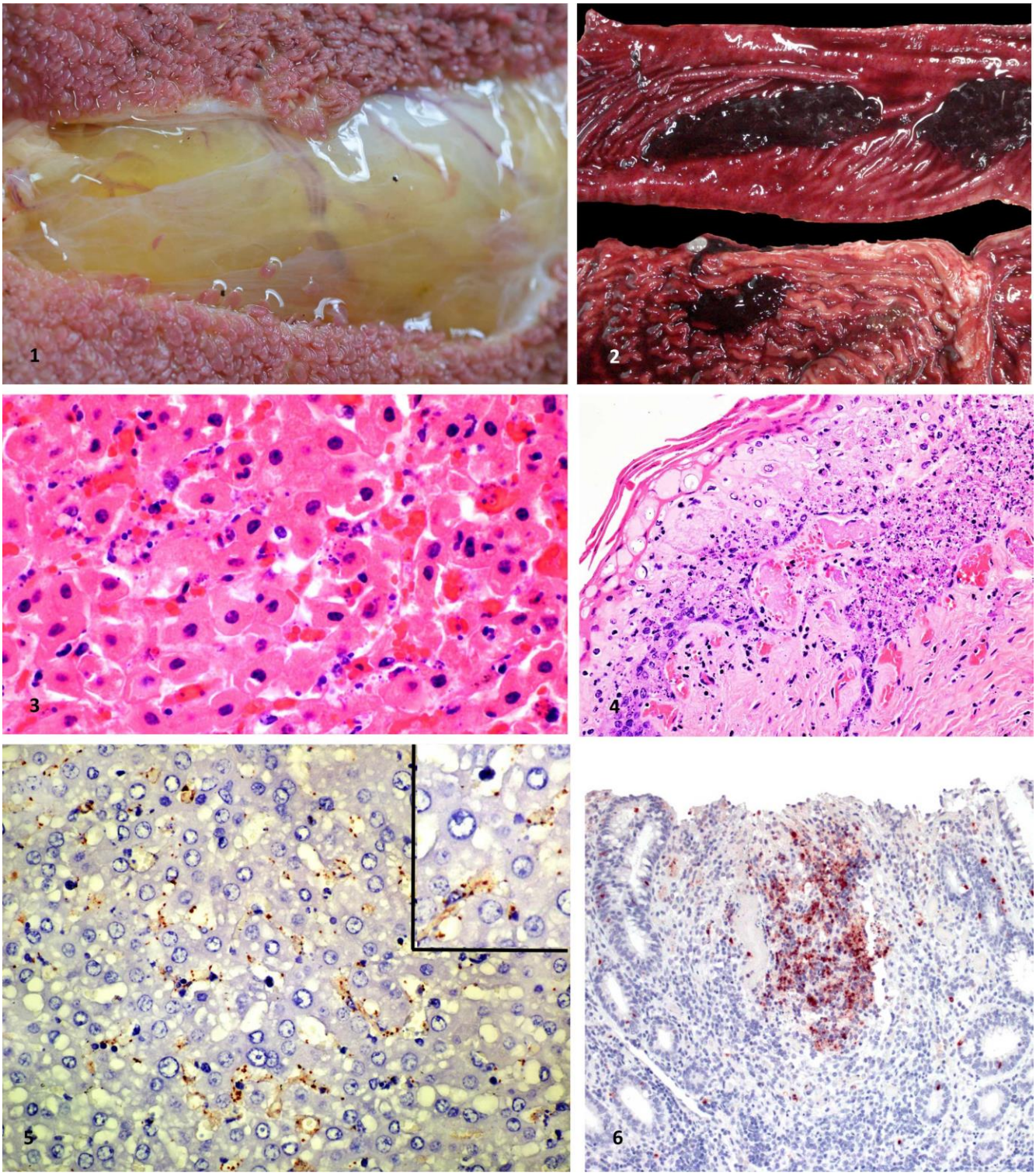
animals was used and no definitive conclusions can be drawn.



Figures 1-6. Calves in intoxicated intraruminally with ground seeds of *Ricinus communis*. **1.** Severe edema of the ruminal mucosa. **2.** Hemorrhage in the lumen of the colon. **3.** Pyknotic and karyorrhectic nuclei in the endothelium of hepatic capillaries. **4.** Mucosal necrosis of the rumen. **5.** Positive immunostaining for activated caspase 3 in the endothelium of hepatic capillaries. There is severe karyorrhexis and the positive immunostaining is therefore distributed irregularly throughout the cytoplasm. Inset: hepatic capillary endothelium showing positive staining for von Willebrand factor VIII **6.** Positive immunostaining to activated caspase 3 in colonic lymphoid aggregates.

The Kupffer cells can be activated by ricin producing reactive oxygen species (8, 12). These molecules are highly reactive with most of the cell components, causing hepatocyte apoptosis or necrosis (16). In this study, the cell death by apoptosis in the liver was confirmed by activated caspase-3 IHC in Kupffer cells and hepatocytes of peri-portal areas.

In vitro studies demonstrated that ricin produce intercellular gaps and apoptosis of endothelial cells, leading to edema (7, 8). In this study, all intoxicated animals had endothelial cell apoptosis in multiple tissues. Apoptosis in these cells was confirmed by activated caspase-3 IHC.

Tubular epithelial cell death was observed in all intoxicated animals. This was also previously described in sheep (2) and dogs (11) naturally intoxicated with *R. communis*. In this study, the negative IHC for activated caspase-3 on renal tubular epithelium indicates that caspase-3 mediated apoptosis was not the mechanism of cell death in these cells. Additional studies are required to determine the mechanism of cell death associated with *R. communis* in these cells.

The strong positivity to activated caspase-3 immunostaining in lymphoid follicles of multiple organs provided evidence that most cells in those locations died by caspase-3 mediated apoptosis. Lymphoid cell apoptosis was observed before in cattle and mice experimentally intoxicated with ricin (6). In our study, although activated caspase-3 IHC was positive in many dead cells of multiple tissues, not all dead cells stained positively for activated caspase-3, indicating that other mechanisms of cell death, in addition to activated caspase-3 associated apoptosis, also occurred.

In previous studies, no gross or microscopic lesions were observed in the gastrointestinal tract of cattle intoxicated with leaves (15) and pericarp (4) of *R. communis*. However, oral intoxication of cattle with seeds of *R. communis* produced mucosal congestion in the small intestine and colon, and submucosal edema in the colon (14). Mild inflammatory infiltration of lymphocytes, macrophages and fewer neutrophils and edema were present in the abomasum and small intestine of cattle spontaneously intoxicated with whole plant of *R. communis* (1). These studies (1, 14) coupled with the results of the current report suggest that the whole seed of *R. communis* is required for alimentary lesions to occur in cattle consuming this plant. These lesions seem to be dose dependent as the alimentary system changes were more severe in animals 3 and 4, which were inoculated with the highest dose of *R. communis* seeds.

In conclusion, oral intoxication of cattle with seeds of *R. communis* produces severe lesions in multiple organs and most cell death in multiple tissues is produced by caspase-3 associated apoptosis.

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