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Preface

We are pleased to publish this second issue of the *International Journal of Poisonous Plant Research* (*IJPPR*). The objectives of *IJPPR* include publishing original research, case reports, and scientific reviews, and sharing new information and technology to improve analysis and diagnosis of plant poisonings. There is currently no journal, electronic or printed, that specifically focuses on poisonous plant research, and *IJPPR* aims to fill this critical role. We've employed an electronic publication system to make the papers as widely accessible as possible on the USDA-ARS Web site at no charge. It is our intent for *IJPPR* to provide a central outlet for poisonous plant research and bring together all disciplines from around the world with a common interest.

This issue provides research information and photographs of plant poisonings from South America and the United States. Special emphasis is given to the scientific review of plants poisonous to cattle in central-western Brazil, including the cover photograph, kindly provided by Dr. Flávia Gontijo de Lima, of Nelore cattle grazing in central Brazil.

The Editors-in-Chief thank those who have assisted in the production of the second issue, particularly Terrie Wierenga, Editorial Assistant, USDA-ARS Poisonous Plant Research Laboratory, Logan, UT, and the following USDA-ARS staff members in Beltsville, MD: Sandy Miller Hays, Director, Information Staff; Jeff Steiner, National Program Leader, Office of National Programs; and Mina Chung, Supervisory Editor, Information Staff. The Editors-in-Chief also thank the *IJPPR* Editorial and Advisory Boards.

Contents

Poisonous Plants Affecting Cattle in Central-Western Brazil

Fernando H. Furlan[1], Edson M. Colodel[2], Ricardo A. A. Lemos[3], Marcio B. Castro[4], Fábio S. Mendonça[5], and Franklin Riet-Correa[6*]

[1] Veterinary Pathology Laboratory, Federal University of Mato Grosso, Campus Sinop, Sinop, Mato Grosso, Brazil
[2]Veterinary Pathology Laboratory, Federal University of Mato Grosso, Campus Cuiabá, Av. Fernando Corrêa da Costa, 2367, Bairro Boa Esperança, Cuiabá, Cuiabá, Mato Grosso, Brazil
[3]Veterinary Medicine Department, Federal University of Mato Grosso do Sul, Campo Grande, MS 79070-900, Brazil
[4]Veterinary Pathology Laboratory, University of Brasília, Brasília, Brazil
[5]Department of Animal Morphology and Physiology, Federal Rural University of Pernambuco, Recife, Pernambuco, Brazil
[6]Veterinary Hospital, Federal University of Campina Grande, Patos, Paraíba, Brazil

*Corresponding author: Franklin Riet-Correa, franklin.riet@pq.cnpq.br

Abstract

Poisonous plants affecting cattle in the Brazilian Central-Western region are reviewed. The most important poisonous plants in the region are *Brachiaria* spp., which cause hepatogenous photosensitization, and *Palicourea marcgravii* and *Mascagnia pubiflora*, which cause sudden death precipitated by exercise. *Enterolobium contortisiliquum*, *Stryphnodendron obovatum*, and *Stryphnodendron fissuratum* are trees whose pods cause digestive signs, photosensitization, and abortion. *Vernonia mollissima* and *V. rubricaulis* cause acute liver necrosis, but *V. rubricaulis* is much more important than *V. mollisima* as a cause of cattle losses. Other less important plants are *Solanum glaucophyllum*, which causes soft tissue calcification; *Ipomoea carnea* subsp. *fistulosa*, which causes nervous signs; *Senna occidentalis* and *Senna obtusifolia*, which cause segmental muscular necrosis; *Tetrapterys multiglandulosa*, which causes cardiac fibrosis, abortion, and neonatal mortality; *Polygala klotzschii,* which causes lymphatic tissue necrosis; and *Pterodon emarginatus*, a tree whose leaves cause liver necrosis. Rare outbreaks of poisoning by *Lantana tiliaefolia*, *Cestrum laevigatum*, *Pteridium caudatum*, and *Crotalaria* spp. also are observed.

Keywords: *Brachiaria* spp., *Enterolobium contortisiliquum*, *Mascagnia pubiflora*, *Palicourea marcgravii*, *Stryphnodendron* spp., *Vernonia rubricaulis*

Introduction

The Central-Western (*Centro-Oeste*) region of Brazil (figure 1), the most important area for cattle production in the country, is composed of the states of Goiás, Mato Grosso, and Mato Grosso do Sul and the Federal District, where Brasília, the Brazilian capital, is located. The Central-Western region represents 18.86 percent of the Brazilian territory. The region is formed by three different biomes: *Cerrado*, *Pantanal*, and, in northern Mato Grosso, the Amazon rainforest. Similar to savannas, the *Cerrado* originally covered the entire area of Goiás and large parts of Mato Grosso and Mato Grosso do Sul. The *Pantanal*, the world's largest plain covered with water, is a tropical wetland that lies mainly in Mato Grosso do Sul and extends into Mato Grosso. The climate is humid subtropical and tropical with annual rainfall of around 800 to 1,600 mm and a rainy season from October to March with a dry

season for the rest of the year. The soils are generally very old, chemically poor, and deep. Large areas of the *Cerrado* and the Amazon rainforest have been deforested for agriculture (soybean, cotton, sunflower, and other crops) and for the cultivation of pastures (mainly *Brachiaria* spp. and *Panicum maximum*) for cattle production. The cattle industry is very important in the Central-Western region of Brazil, with nearly 70 million head of mainly beef cattle, which represents 36 percent of the Brazilian cattle population. The objective of this paper is to review toxic plants affecting cattle in the Brazilian Central-Western region.

Figure 1. Map of Brazil showing the different regions, Brasília (★), and the states of Goiás, Mato Grosso, and Mato Grosso do Sul.

Brachiaria spp.

Brachiaria spp. (Poaceae) are the most important toxic plants in the Brazilian Central-Western region. *Brachiaria decumbens* cv. Ipean, originally from Africa, was first introduced to Brazil in 1962. In 1972, *B. decumbens* cv. brasilisk was introduced from Australia and spread rapidly throughout the region (Seiffert 1980). *B. brizantha* cv. Marandú, which has gradually replaced *B. decumbens,* was introduced in the 1980s. *B. humidicola* was introduced from Africa in 1983. Other species of *Brachiaria* include *B. purpurascens*, *B. dictyoneura*, *B. ruziziensis*, *B. radicans*, *B. extensa*, *B. plantaginea*, *B. dura*, and *B. milliformis* (Seifert 1980). There are about 60 million hectares of cultivated pastures in the *Cerrado*. Of this total, 51 million hectares consist of *Brachiaria* spp., approximately 30 million hectares of *B. brizantha*, 15 million hectares of *B. decumbens*, and 6 million hectares of *B. humidicola* and other *Brachiaria* (Macedo 2005). Most outbreaks of poisoning are caused by *B. decumbens* (Camargo et al. 1976; Döbereiner et al. 1976b; Nobre and Andrade 1976; Fagliari et al. 1983, 1993b; Lemos et al. 1996, 1997; Brum et al. 2007; Mustafa 2009; Souza et al. 2010). Outbreaks caused by *B. brizantha* (Mustafa 2009, Lemos et al. 2011, Riet-Correa et al. 2011) are less frequent. Few outbreaks have been reported from *B. ruziziensis* (Nazário et al. 1985, Purchio et al. 1988) but this species is rarely used in pastures of the region. Poisoning by *B. humidicola* has been reported in sheep (Schenk and Schenk 1983), buffalo (Láu 1990), and horses (Barbosa et al. 2006) but not in cattle.

The toxicity of *Brachiaria* spp. is due to lithogenic steroidal saponins, protodioscin being the main saponin found in the different species (Brum et al. 2007, 2009; Santos Jr., 2008; Mustafa 2009; Castro et al. 2011). A toxicity threshold is yet to be established, but for sheep, it was suggested that pastures with saponin concentrations of more than 1 percent cause toxicity in naive sheep (Riet-Correa et al. 2011). There is no information on saponin concentrations in pastures causing poisoning in cattle. In general, saponin concentrations are higher in growing plants, but outbreaks occur throughout the year, probably due to unexplained rises in saponin concentrations in the plant. Most data show that sprouting pastures have higher saponin concentrations than mature ones (Santos Jr. 2008, Castro et al. 2011), and green leaves contain more saponin than mature or senescent leaves (Barbosa-Ferreira et al. 2011).

Brachiaria poisoning has been reported in cattle, sheep, and goats. Sheep are more susceptible to poisoning than cattle (Riet-Correa et al. 2009, Lemos et al. 2011). Young cattle up to 2 years of age are more frequently affected, but adult cattle may also be affected. The poisoning occurs mainly in calves near weaning or just weaned (Nobre and Andrade 1976, Fagliari et al. 1993a, Souza et al. 2010), but suckling animals up to 30 days old also are affected (Fagliari et al. 1983, Lemos et al. 1996).

Castro et al. (2011) demonstrated that there are differences in susceptibility between animals of the same species. Sheep raised on *Brachiaria* spp. pastures are less susceptible to poisoning than naive sheep raised on other pastures (Castro et al. 2011), and it has been suggested that resistance is genetic (Riet-Correa et al. 2011). In cattle, *Brachiaria* spp. poisoning was more frequent in the years after the introduction of *B. decumbens* (1960 to 1980). Subsequently, outbreaks were less frequent, apparently due to the death of susceptible animals or to the development of poisoning resistance. Another possibility is that the decrease in the number of

outbreaks is a result of the replacement of *B. decumbens*, which is more toxic, by the less toxic *B. brizantha* and *B. humidicola* (Castro et al. 2011, Riet-Correa et al. 2011). It has also been suggested that buffalo and probably some sheep are resilient, i.e., when poisoned these animals show histologic lesions and high GGT serum concentrations but they do not show clinical signs (Saturnino et al. 2010, Castro et al. 2011, Riet-Correa et al. 2011).

Poisoning can occur at any time of the year and at any stage of the plant growth (Castro et al. 2011, Souza et al. 2011), but some authors mention a higher frequency of the poisoning during pasture sprouting (Riet-Correa et al. 2009, Castro et al. 2011). Frequency of the poisoning varies; in 29 cattle outbreaks in Mato Grosso do Sul, the morbidity rate ranged from 0.2 to 50 percent, and the fatality rate from 44.4 to 100 percent (Souza et al. 2010).

In cattle, main clinical signs are hepatogenous photosensitization with dermatitis mainly on the muzzle, ears, flanks, perineum, udder, and in areas of white skin. More acute cases show edema of the brisket and ears or other body parts, and a common finding in chronic cases is thickening and scar retraction of the ears, which are deformed and crooked (Fagliari et al. 1993b, Lemos et al. 1997, Souza et al. 2010). A clinical condition characterized by progressive weight loss and death after a few months without photosensitization has been reported in cattle over 2 years of age grazing *B. decumbens* (Riet-Correa et al. 2002, Souza et al. 2010).

In cattle, a subclinical disease characterized by lower productivity has also been reported (Fagliari et al. 1993b, Fioravanti 1999, Moreira et al. 2009a). However, Castro et al. (2011) found no significant differences in weight gains when young sheep raised on *Brachiaria* pastures were introduced to *B. brizantha, B. decumbens*, or *Panicum maximum* pastures. Sheep introduced to an *Andropogon gayanus* pasture showed lower weight gain than the others. In the same experiment naive sheep introduced to *B. decumbens* pastures showed clinical signs of poisoning.

Increased serum activities of gamma glutamyl transferase (GGT) and aspartate aminotransferase (AST) and increased serum concentrations of total, direct, and indirect bilirubin have been detected in natural and experimental *Brachiaria* spp. poisoning in cattle and sheep (Fagliari et al. 1994, Fioravanti 1999, Mendonça et al. 2008, Santos Jr. 2008, Saturnino et al. 2010, Castro et al. 2011). Increased serum GGT levels are the best predictors of the onset of the poisoning, tending to remain high for a longer period (Fagliari et al. 1994, Santos Jr. 2008, Saturnino et al. 2010, Castro et al. 2011). However, increased AST and GGT levels did not correlate with the severity of poisoning (Santos Jr. 2008, Saturnino et al. 2010, Castro et al. 2011).

The main macroscopic lesions, in addition to dermatitis, are jaundice, an enlarged yellowish or brownish liver, a distended gallbladder with edematous wall, subcutaneous yellowish edema, ascites, hydropericardium, and hydrothorax. On histologic examination, the liver shows vacuolation, swelling or necrosis of individual hepatocytes, bilestasis, lymphoplasmocytic cholangitis and pericolangitis and, in chronic cases, a varying degree of periportal fibrosis. The most characteristic lesions of *Brachiaria* spp. poisoning are refringent crystals or negative images of these crystals in the bile ducts, macrophages, and hepatocytes (Lemos et al. 1996, 1997; Driemeier et al. 2002; Santos Jr. 2008), and the occurrence of macrophages with foamy cytoplasm, sometimes containing crystals (Lemos et al. 1997; Driemeier et al. 1998, 1999, 2002; Gomar et al. 2005; Santos Jr. 2008; Moreira et al. 2009b).

To control poisoning, cattle should be removed from toxic pastures. The only treatment is symptomatic. It is very important to keep the animals in the shade and provide them with food and water. In the future, main preventive measures should be based on the selection of resistant or resilient animals and on the development of *Brachiaria* species or varieties with low saponin concentrations.

Palicourea marcgravii

Palicourea marcgravii (Rubiaceae) (figure 2) is the most important native toxic plant in Brazil. Until the 1990s, *P. marcgravii* was the most important toxic plant in the Central-Western region. The plant is found mainly in the native forest, and it cannot survive in pastures without shade. With the deforestation and the substitution of native forests by *Brachiaria* spp. pastures, the poisonings have become less frequent, but they still remain a concern in Goiás, Mato Grosso (mainly in the north of the state), and in the Federal District, but not in Mato Grosso do Sul (Tokarnia and Döbereiner 1986, Tokarnia et al. 1990). Because it is palatable and very toxic, sudden deaths occur in all places where the plant is found. The toxic compound of *P. marcgravii* is fluoroacetate (Oliveira 1963, Krebs et al. 1994), and doses of 0.6 to 2 g of fresh leaves per kg bodyweight (g/kg) are lethal to cattle (Tokarnia and Döbereiner 1986, Tokarnia et al. 1990).

Figure 2. *Palicourea marcgravii*.

In most cases, clinical signs are hyperacute, and they appear when the animals are exercising. The characteristic signs are loss of balance, ataxia, tachycardia, tachypnea, muscular tremors, and falling down. Some animals rise but immediately fall down again, staying in lateral recumbence and pedaling in an attempt to stand up. Nervous signs like opisthotonous and bruxism also are observed. These severe signs last, in most cases, 1 to 10 minutes before death. In some animals, early clinical signs are mild, and they consist of reluctance to walk or moving slowly, lying down frequently and for long periods, heart palpitations with dilated and visible pulsing jugular vein, and dyspnea. In these cases, death occurs after 12 to 55 hours. Some animals are found dead without exercise. Affected animals rarely recover (Tokarnia and Döbereiner 1986, Tokarnia et al. 1990). There are no significant gross lesions. Histologically, nearly 60 percent of the animals had necrosis and hydropic degeneration of the epithelium of distal convoluted tubules of the kidney (Tokarnia and Döbereiner 1986).

Clinical signs linked to exercise and to the presence of the plant are suggestive of the diagnosis. The histologic lesion of the kidneys is characteristic but is not observed in all cases. Other plants cause sudden death in Brazil with similar signs and lesions, but only *P. marcgravii* and *Amorimia* (*Mascagnia*) *pubiflora* are found in the Central-Western region of Brazil.

Poisoning from these plants is very difficult to control except by the use of fences to isolate areas with the plant. Under an agreement with CSIRO, Australia, and the USDA-ARS Poisonous Plant Research Laboratory in Logan, UT, our research group in Brazil (Institute of Science and Technology for the Control of Plant Poisoning), isolated fluoroacetate-degrading microorganisms to be used as a rumen inoculate to reduce losses from fluoroacetate-containing plants (Camboim et al. 2012).

Amorimia (*Mascagnia*) *pubiflora*

Amorimia pubiflora (Malpighiaceae) (figure 3) is a very important toxic plant in Mato Grosso do Sul, Goiás, and Mato Grosso (Tokarnia and Döbereiner 1973, Tokarnia et al. 1990, Lemos et al. 2011). Most outbreaks occur during the dry period. Morbidity varies from 1 to 3.5 percent and case fatality rate is nearly 100 percent but some animals recover if they do not exert themselves (Tokarnia and Döbereiner 1973, Tokarnia et al. 1990, Lemos et al. 2011). The toxic compound in *A. pubiflora* is unknown, but it is probably fluoroacetate. Clinical signs of poisoning by *A. pubiflora* are very similar to those mentioned in *P. marcgravii* poisoning. There are no significant lesions at necropsy. Histologic lesions of vacuolar degeneration and necrosis of distal convoluted tubules of the kidney were observed in 66 percent of cattle poisoned experimentally (Tokarnia and Dobereiner 1973) and also in some spontaneous cases (Ricardo Lemos, 2011, unpublished).

Figure 3. *Amorimia* (*Mascagnia*) *pubiflora*

The control of poisoning by *A. pubiflora* is difficult. Hand removal of the plant is a good control measure for small areas, but it is not feasible in most areas. *Amorimia* spp. have a persistent root crown that facilitates regrowth after removal of the plant or use of herbicides. The use of fences to isolate areas with the plant is a good control measure for some farms. Goats develop considerable resistance after the continuous ingestion of small amounts of *Amorimia* (*Mascagnia*) *rigida*, another poisonous plant, and that resistance is transferred by transfaunation of the ruminal content, suggesting that resistance is due to ruminal microorganisms that degrade fluoroacetate (Franklin Riet-Correa et al., 2011, unpublished data).

Enterolobium contortisiliquum and *Stryphnodendron* spp.

In the Central-Western region of Brazil, there are a group of leguminous trees belonging to the family Fabaceae, subfamily Mimosoideae, including *Enterolobium contortisiliquum* (= *Enterolobium timbouva*) (figure 4) (Tokarnia et al. 1960, 1999; Grecco et al. 2002; Mendonça et al. 2009; Lemos et al. 2011), *Stryphnodendron obovatum* (Brito et al. 2001a,b), and *Stryphnodendron fissuratum* (figure 5) (Ferreira et al. 2009) that produce pods during the dry season whose consumption has been associated with digestive signs, photosensitivity, and abortion in cattle. Poisoning by *E. contortisiliquum* pods has been frequently diagnosed in Mato Grosso (Tokarnia et al. 1960, 1999; Grecco et al. 2002; Mendonça et al. 2009) and Mato Grosso do Sul (Lemos et al. 2011). The intoxication occurs when the animals ingest the fallen pods from August to November. In field outbreaks of poisoning by *E. contortisiliquum*, the main clinical signs are digestive signs (mainly diarrhea), photosensitization, and abortion, but experimentally only digestive signs (Tokarnia et al. 1960, 1999; Grecco et al. 2002; Mendonça et al. 2009; Lemos et al. 2011) and mild photosensitization (Grecco et al. 2002, Lemos et al. 2011) have been produced. The main histologic lesions are degeneration and necrosis of the epithelium of the forestomachs with formation of intraepithelial vesicles. In the liver, swelling, vacuolization, and individual necrosis of hepatocytes, and in some cases, discrete proliferation of epithelial bile duct cells are observed. The abortive properties of *E. contortisiliquum* were demonstrated in guinea pigs (Bonel-Raposo et al. 2008) but not in cattle (Tokarnia et al. 1999, Lemos et al. 2011). Cattle become tolerant to the toxicity of the pods if they ingest successive non-lethal doses (Tokarnia et al. 1999). Triterpenoid saponins (Carvalho 1981) pathogenic for guinea pigs (Bonel-Raposo et al. 2008) were isolated from *E. gummiferum* pods. Mimaki et al. (2003, 2004) identified enterolosaponin A and contortisilioside B, which were toxic to macrophages, and contortisilioside A and C, toxic to macrophages and murine lymphoma cells.

Figure 4. *Enterolobium contortisiliquum*. The cattle are consuming pods (inset) of the tree.

Stryphnodendron obovatum is found in the three states of the Central-Western region of Brazil, but outbreaks of the poisoning have been reported mainly in Mato Grosso (Brito et al. 2001a). The experimental administration of *S. obovatum* to cattle caused anorexia, liquid feces, abdominal distention without tympany, regurgitation, ruminal hypotonia, ruminal acidosis, gastrointestinal colic, drooling, apathy, weight loss, and erosions and ulcers of the oral mucosa. Mild lesions of the skin due to photosensitization also were observed (Brito et al. 2001a). Main lesions were degeneration and necrosis of the digestive epithelium with formation of intraepithelial vesicles and pustules in the oral cavity, esophagus, and forestomachs (Brito et al. 2001b). The administration of *S. obovatum* causes similar digestive signs in pregnant cows, and three out of seven cows aborted (Tokarnia et al. 1998).

Outbreaks of poisoning by *S. fissuratum* (figure 5) were reported in Goiás, Mato Grosso, and Mato Grosso do Sul from cattle consuming pods of this tree from July to November (Ferreira et al. 2009, Lemos et al. 2011). The clinical manifestation period varied from 24 hours to 10 days after consumption of the plant, and the morbidity and case fatality rates were 0.9-25 percent and 15-100 percent, respectively. The main clinical signs in the

Figure 5. *Stryphnodendron fissuratum*. A) tree; B) leaves and pods; C) a branch of the tree with leaves and pods.

spontaneous poisoning were apathy, anorexia, aggressiveness, jaundice, drooling, incoordination, dysmetria, retraction of the abdomen, uneasiness, pasty black feces with strings of mucus or blood, diarrhea, edema of the dewlap, and hepatogenous photosensitization. At necropsy, jaundice, edema of the subcutaneous tissue (mainly of the cervical region), hemorrhages of serous membranes, ascites and hydrothorax, edema of the mesentery, perirenal edema, increased size of liver and kidney, reddening of the ruminal mucosa, and abomasum ulcers were observed. The main histologic lesions are degeneration and necrosis of the epithelium of the forestomachs with formation of intraepithelial vesicles, swelling, vacuolization, and individual necrosis of hepatocytes, and mild nephrosis (Ferreira et al. 2009). Experimentally, digestive signs were reproduced by the administration of pods to two bovines, but photosensitization was not observed before the animals died. Farmers in the state of Mato Grosso do Sul have observed abortion from poisoning by *S. fissuratum*, and the abortive properties of the pods were demonstrated in goats (Alburquerque et al. 2011) and cows (Ricardo Lemos, 2011, unpublished). Triterpenoid saponins were isolated from *S. fissuratum* (Haraguchi et al. 2006, Yokosuka et al. 2008)

To control poisoning by *Stryphnodendron* spp. and *E. contortisiliquum*, it is necessary to prevent the ingestion of the pods by cattle. However, frequently the ingestion of pods of *E. contortisiliquum* by cattle causes no clinical signs, which can be due to the development of resistance, to the ingestion of low doses, or to both. In recent experiments, no significant differences in toxicity were found between plants from different regions (Franklin Riet-Correa and Ricardo A. Lemos, 2011, unpublished data).

Vernonia mollissima and *Vernonia rubricaulis*

Vernonia mollissima and *V. rubricaulis* (Asteraceae) (figure 6) have been reported as toxic for cattle in Mato Grosso do Sul (Döbereiner et al. 1976, Tokarnia and Döbereiner 1982), but since these reports, no more outbreaks of poisoning by *V. mollisima* have been reported in this region (Lemos et al. 2011). In contrast, poisoning by *V. rubricaulis* is an important cause of death in cattle in Mato Grosso do Sul (Brum et al. 2002, Lemos et al. 2011).


Figure 6. *Vernonia rubricaulis*.

Poisoning by *V. mollisima* occurs mainly when the plant is sprouting or during periods of forage scarcity from August to September (Dobereiner et al. 1976a). Poisoning by *V. rubricaulis* occurs annually in the late dry or early wet season (August to December), when the plant is green and other forages are dry. Some conditions are important for the occurrence of the intoxication: management practices that encourage sprouting of the plant (e.g. mechanical removal or fire); exposing naive animals to the plant either by transporting them from other ranches or moving them into unfamiliar pastures; and excessive stocking rates on pastures. Morbidity varied from 2 to 21 percent, and case fatality is

nearly 100 percent (Brum et al. 2002, Lemos et al. 2011).

Clinical signs are characterized by apathy, tremors, dry muzzle (dehydration), dry feces with blood, and aggressive behavior. Histological findings are severe coagulation necrosis, mainly in the centrilobular region of the liver, and hemorrhages, occasionally affecting the whole lobule. Cases with massive necrosis of the liver and bleeding are frequent (Brum et al. 2002, Lemos et al. 2011). The toxic compound of the plant is unknown.

To prevent intoxication, it is necessary to avoid grazing *V. rubricaulis* during sprouting of the plant after burns, mowing, and rotational grazing. Caution is necessary when animals are moved from areas where *V. rubricaulis* does not exist into paddocks invaded by this plant.

Solanum glaucophyllum (=*Solanum malacoxylon*)

Solanum glaucophyllum (Solanaceae) (figure 7) is a toxic plant of the Brazilian Pantanal. It causes a chronic condition characterized by soft tissue calcification, hypercalcemia, hypercalcitoninism, and osteopetrosis. The disease occurs most frequently from June to October during the dry season and mainly during periods of forage scarcity. The plant is commonly found in low-lying flood plains and in marshy areas near rivers and streams (Döbereiner et al. 1971, Tokarnia and Döbereiner 1974, Lemos et al. 2011), but in one outbreak, the area was deforested to plant a pasture, and *S. malacoxylon* invaded this area in large amounts (Lemos et al. 2011). In three outbreaks reported recently, morbidity varied from 1.7 to 3.75 percent and lethality from 2 to 100 percent (Lemos et al. 2011). As the plant is commonly found in the Brazilian Pantanal, the intoxication is known by farmers and practitioners, and cases of intoxication are probably underreported (Lemos et al. 2011). The poisoning was reported also in buffalo from January to March during the rainy season (Santos et al. 2011).

Clinical signs are characterized by progressive weight loss, stiff gait and lameness, tucked-up abdomen, kyphosis, and heart murmurs. Animals tend to remain recumbent and have a difficult time standing up. Other signs are dyspnea and an increase in the size and rigidity of the arteries, particularly in the facial arteries, and in the iliac arteries by rectal palpation. The clinical course is chronic, and death can occur up to 4 months from extreme malnutrition and cachexia if animals are not removed from pastures where the plant occurs (Döbereiner et al. 1971, Tokarnia and Döbereiner 1974).

Figure 7. *Solanum glaucophyllum*.

The lesions observed at necropsy are characterized by thick, hard, and inelastic arterial walls, excluding the pulmonary artery. The tunica intima of the arteries appears wrinkled and covered with mineralized deposits. There also is calcification of the bicuspid and aortic valves and occasionally the endocardium, and mineralization in the lungs, especially on the borders of the diaphragmatic lobes. The renal cortices have focal white areas of mineralization in the cortex and white streaks of mineralization in the medulla. Histologically, there is edema and fragmentation of elastic fibers in arteries of diverse organs, with granular deposits and mineral plaque. There also is calcification of tendons and ligaments (Döbereiner et al. 1971, Tokarnia and Döbereiner 1974, Lemos et al. 2011). The toxins in *Solanum malacoxylon* are derived glycosides of 1,25-$(OH)_2$ D_3 (calcitriol) (Wasserman 1978).

There is no treatment. If animals are removed as soon as they show clinical signs, they can clinically recover and gain weight, but the lesions are not reversible. Avoiding grazing of cattle in areas severely invaded by the plant is the only way to prevent poisoning.

Ipomoea carnea subsp. *fistulosa*

I. carnea subsp. *fistulosa* (Convulvulaceae) (figure 8) is very common in the Brazilian Pantanal and farmers in the region reported the occurrence of poisoning in cattle during the dry period. The disease occurs when there is low forage availability. Most animals recover if they are removed from the paddocks invaded by this plant. One outbreak of poisoning by this swainsonine-containing plant was

reported in cattle in the Pantanal floodplains from June to September 2006 (dry season) in an area severely invaded by *I. carnea* with low forage availability (Antoniassi et al. 2007). In three other outbreaks between 2008 and 2009, some animals developed a preference for *I. carnea* and continued eating it even in the rainy season, when there was greater availability of forage in the pasture, which resulted in intoxication and deaths during this period (Fábio S. Mendonça, 2011, unpublished). Neurological signs, characteristic of cerebellar and brainstem alterations, were mainly hypermetria, ataxia, and intention tremors. Severe weight loss was also observed. No gross lesions were observed at necropsy. The main histologic lesions were vacuolation of the pericaryon of neurons and in the cytoplasm of epithelial cells of the thyroid, kidney, and pancreas. One affected bovine removed from the area invaded by *I. carnea* subsp. *fistulosa* recovered clinically within 15 days (Antoniassi et al. 2007).

Figure 8. *Ipomoea carnea* subsp. *fistulosa.*

Senna occidentalis and *Senna obtusifolia*

Senna occidentalis (figure 9) and *Senna obtusifolia* (figure 10) (Fabaceae) are common weeds in Central-Western Brazil that occasionally cause poisoning in cattle grazing these plants. In two outbreaks of intoxication by *S. occidentalis* when it was in seed in Mato Grosso do Sul, morbidity varied from 6.4 to 17 percent and lethality was 100 percent, but in another outbreak, morbidity was 62 percent. This high morbidity probably was due to the high stocking rate in a paddock severely invaded by *S. occidentalis*, with low forage availability due to previous grazing (Lemos et al. 2011). Poisoning by contamination of grains by seeds of *S. occidentalis* as observed in different species in other Brazilian regions has not been reported in the Central-Western region. Poisoning by *S. obtusifolia* has been diagnosed recently in Mato Grosso, and the poisoning was reproduced experimentally by the administration of daily doses of 15 g of green leaves with pods per kg BW for 6 days (Fernando Furlan, 2011, unpublished data).

Figure 9. *Senna occidentalis*.

Main clinical signs are diarrhea, muscle weakness, ataxia of the hind limbs, restlessness, and recumbency, followed by death after a clinical manifestation period of 4 to 12 days. Occasionally, in some outbreaks, some animals recovered after being recumbent for some days. At necropsy, pale areas of some muscles and bleeding and congestion of the fascia are observed. The bladder contains dark urine, and in the case of *S. occidentalis* poisoning, seeds of this plant can be observed in the reticulum. Occasionally, pale areas in the myocardium and passive chronic liver congestion are observed. Main histological findings are segmental necrosis of the muscles. Centrilobular necrosis and dilatation of the renal tubules with presence of hyaline casts are occasionally observed. To prevent intoxication, it is necessary to avoid grazing animals in paddocks

Figure 10. *Senna obtusifolia*. Hungry cows grazing in a pasture severely invaded by *S. obtusifolia* (insets) in a paddock where the poisoning occurred.

severely invaded by *S. occidentalis* or *S. obtusifolia* and avoid feeding grain contaminated with *S. occidentalis* seeds.

The toxins in the plants have not been completely isolated or identified, but anthraquinones (Lewis and Shibamoto 1989) and a compound named diantrons (Haraguchi et al. 1996) were suggested as possible toxins in *Senna* spp.

Pterodon emarginatus

Pterodon emarginatus (Fabaceae) (figure 11) is a tree found in the three states in the Brazilian Central-Western region. The intoxication seen in cattle in Mato Grosso (Arruda et al. 2008) and Mato Grosso do Sul (Lemos et al. 2011) occurs when cattle ingest leaves of trees that have been cut for wood. Farmers claim that the poisoning is frequently observed between July and September (dry season) after windstorms. The poisoning causes hepatogenous photosensitization and hepatic encephalopathy with

Figure 11. *Pterodon emarginatus*. Inset: leaves and pod.

nervous signs including incoordination, apparent blindness, and depression. The death occurs after a clinical manifestation period of 12 to 72 hours. The liver is enlarged with increased lobular pattern and a diffuse mottled appearance with red areas intercalated with yellow areas. Severe hemorrhages in the abdominal and thoracic serosa also were observed. The main histologic lesion is centrilobular (periacinar) liver necrosis. The poisoning was reproduced experimentally in bovines after the administration of 3 g of fresh leaves/kg BW, but the toxicity seems to be very variable (Edson M. Colodel and Ricardo Lemos, 2011, unpublished data). The toxic compound of *P. emarginatus* is unknown.

Polygala klotzschii

Polygala klotzschii (Polygalaceae) is a small spiny shrub reported as toxic for cattle in the 1970s in a small area of Mato Grosso do Sul (municipalities of Amambaí, Guatemí, Anaurilândia, and Nova Andradina). No more outbreaks have been reported since then, and the poisoning is apparently of reduced importance. The plant is low in palatability, and the intoxication occurs mainly during the dry season when forage is scarce. Cattle of all ages are affected, morbidity is variable, and case fatality is high (Tokarnia et al. 1976). The toxic compound is 5-metoxi-podophyllotoxin, which belongs to the group of the podophyllins. The intoxication is acute and characterized by anorexia, salivation, severe depression, diarrhea, incoordination, and death within 10 to 38 hours. Gross lesions are ascites, hydrothorax, petechial hemorrhages in the trachea, endocardium and gut, enlarged and reddish lymph nodes, distention of the gall bladder, and congestion of lung, liver, kidney, and brain. Omasal content is dry, and the plant can be found in the rumen. The most characteristic histologic lesion is diffuse necrosis of lymphocytes, mainly in the germinative centers of the follicles of the spleen, lymph nodes, Payer patches, and other lymphatic tissues (Tokarnia et al. 1976).

Tetrapterys multiglandulosa

Tetrapterys multiglandulosa (Malpighiaceae) (figure 12) is a well-known toxic plant in southeastern Brazil causing three clinically different diseases, which can occur in isolation or together: a perinatal form with abortion or neonatal death, a nervous form with vacuolation (status spongiosus) of the nervous system, and a cardiac form with fibrosis of the heart,

causing sudden death or congestive heart failure. Poisoning by *T. multiglandulosa* was diagnosed twice on a farm in Mato Grosso do Sul (Carvalho et al. 2006). In the first case, a herd of 290 pregnant cows was affected; 7 cows (2.4%) died of cardiac insufficiency, and 230 cows (79.3%) aborted or delivered weak calves that died after parturition. Forty days later on the same farm, only non-pregnant heifers were affected by neurological disturbances and cardiac insufficiency: 9 of 285 heifers showed clinical signs and died. Main lesions in cows and calves were necrosis and fibrosis of the myocardium, chronic passive congestion of the liver, pulmonary edema, and status spongiosus in the white matter of the brain (Carvalho et al. 2006).

Figure 12. *Tetrapterys multiglandulosa*. Insets: fruits (top) and flowers (bottom).

Other Toxic Plants

One outbreak of poisoning by *Lantana tilaefolia* (Verbenaceae) was reported in Mato Grosso in a herd of 400 cows introduced to a paddock severely invaded by this plant. Sixty animals showed severe photosensitization and jaundice, and 59 died. Clinical signs were observed within 10 days after the introduction of the cows to the paddock, and the clinical manifestation period was between 1 and 8 days. *Lantana* spp. contains the triterpenes Lantadene A and Lantadene B, which affect the periportal hepatocytes causing colestasis. The disease was produced experimentally by the administration of a single dose of 30 g/kg BW of fresh *L. tilaefolia* in one calf and 4 daily doses of 4 g/kg BW in another (Tokarnia et al. 1984).

Poisoning by *Cestrum laevigatum* (Solanaceae) has been reported in southern Mato Grosso do Sul. The disease occurs in hungry animals mainly in the presence of sprouting plants after cutting or fire or after exposure of naive animals to the plant (Purisco et al. 1998). The poisoning is characterized by acute liver necrosis. Clinical signs and pathology, reported elsewhere, are similar to other plants causing acute liver necrosis (Riet-Correa et al. 2009). Prevention or reduction of losses is accomplished by eradicating the plants, by avoiding overgrazing in invaded areas, or by the use of fences in areas invaded by the plant (Purisco et al. 1998). The toxin contained in *C. laevigatum* in unknown, but this plant probably contains kaurene glycosides similar to those reported in *Cestrum parqui* (Oelrichs et al. 1994).

An outbreak of acute poisoning by *Pteridium caudatum* (Polypodiaceae) was observed in Mato Grosso do Sul in the Amazonic Region in a herd of 306 cattle. After 40 days grazing in a degraded pasture severely invaded by this plant, 22 bovines were affected, and 20 died after a clinical manifestation period of nearly 3 days. Clinical signs were depression, intolerance to exercise, pale mucosa, fever (41-42ºC), hemorrhages, and increased time for blood coagulation. Hemorrhages were observed at necropsy and on histologic examination there was severe aplasia of the bone marrow (Fernando Furlan, 2011, unpublished).

In Mato Grosso do Sul, three outbreaks of liver fibrosis and interstitial pneumonia in cattle, similar to those observed on pyrrolizidine alkaloids poisoning, were associated with the ingestion of *Crotalaria* spp. (Leguminoseae), but the species of the plant was not identified (Lemos et al. 2011).

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Ethnobotanical Study of Plants Poisonous to Cattle in Eastern Colombia

Paola Torres[1], Gonzalo J. Diaz[2], Edgar Cárdenas[2], and María C. Lozano[2*]

[1]Faculty of Veterinary and Animal Husbandry, National University of Colombia, Bogotá, Colombia
[2]Faculty of Science, Department of Pharmacy, National University of Colombia, Bogotá, Colombia

[*]Corresponding author: Maria C. Lozano, mclozanoa@unal.edu.co

Abstract

A survey of the plants considered to be poisonous to cattle was conducted in eastern Colombia by veterinarians and biologists. This area is characterized by high flora diversity and a large cattle population (approximately 5 million bovines, or 19 percent of the total population in Colombia). Livestock producers on 148 farms were queried about plants empirically known to be poisonous and the effects associated with these plants. Nineteen plants were recognized as toxic by more than two respondents, with *Enterolobium cyclocarpum* the most frequently pointed out by the producers. The plant families and number of species identified as poisonous were Apocynaceae (six), Bignonaceae (three), Verbenaceae (two), Sapindaceae (two), Cyperaceae (one), Heliconaceae (one), Fabaceae (one), Phytolaccaceae (one), and Solanaceae (one). Frequently reported toxic effects were sudden death and photosensitization.

Keywords: Apocynaceae, Colombia, *Enterolobium cyclocarpum*, ethnobotany, poisonous plants

Introduction

Cattle production is an important economic activity in Colombia, which has the ninth largest cattle population in the world (FAO 2010). Cattle are raised extensively on native rangelands, where animals have access to a diverse flora. The flora of Colombia has the second highest biodiversity of plants worldwide (Romero et al. 2008); thus the potential for plant poisoning is high. In the eastern plains of Colombia, where animal husbandry is practiced extensively, significant economic losses to the livestock industry have been reported from a number of toxic plants.

Toxic plants affect cattle in many ways. Signs include muscular weakness, weight loss, gastrointestinal and neuromotor abnormalities, photosensitivity, bleeding, abortions, and birth defects. Sudden death can occur without the presentation of clinical signs (Plumlee 2004). Clinical signs have been attributed to various toxic compounds, including nitrates, oxalates, cyanogenic glycosides, cardiac glycosides, monofluoracetic acid, ptaquiloside, and alkaloids (Frohne and Pfander 2005, Diaz 2010).

The negative economic impact on livestock farms caused by toxic plants is attributed to losses due to livestock deaths, decreased performance parameters, and the need for preventive or therapeutic interventions (Nielsen 1988, Riet-Correa and Medeiros 2001). A first approach to avoid the negative impact of toxicoses on cattle farms is the identification of plant species that can affect animals, using ethnobotany as a source of information. By surveying livestock producers, this study describes the toxic plants that are frequently recognized in the Colombian Orinoco region, where 19 percent of the cattle population in the country is located.

Materials and Methods

The study was conducted in the Colombian Orinoco, specifically in the states of Meta and Casanare, where the largest number of cattle are concentrated. Rainfall in this region may exceed 3,000 mm/year, with a rainy season occurring from April to November, and a dry season from December to

March. The temperature ranges from 22 to 27°C. The relative humidity is above 80 percent during the rainy season and between 60 and 65 percent during the dry season (Rippstein et al. 2001). Meta and Casanare are located between 1° 36' 52" and 6º 20' 45" North latitude and 69° 50' 28" and 74° 53' 57" West longitude, comprising a total of 48 municipalities (29 in Casanare and 19 in Meta), 35 of which were included in this study (figure 1). The total area encompasses approximately 131,000 km^2.

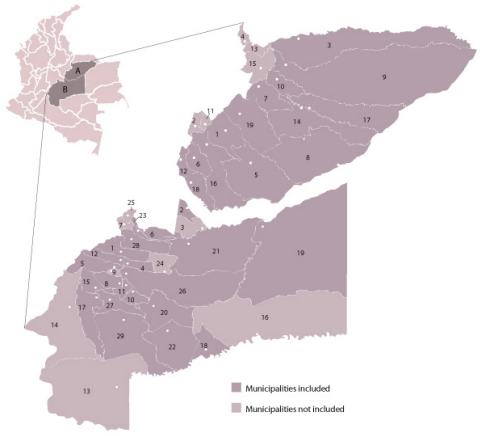

Figure 1. Map of the region considered in this study. The Departments (A and B) and municipalities include: A—Casanare: 1. Aguazul, 2. Chámeza, 3. Hato Corozal, 4. La Salina, 5. Maní, 6. Monterrey, 7. Nunchía, 8. Orocué, 9. Pore, 10. Recetor, 11. Sabanalarga, 12. Sácama, 13. San Luis de Palenque, 14. Támara, 15. Tauramena, 16. Trinidad, 17. Villanueva, 18. Yopal. B—Meta: 1. Acacías, 2. Barranca de Upía, 3. Cabuyaro, 4. Nueva Castilla, 5. Cubarral, 6. Cumaral, 7. El Calvario, 8. El Castillo, 9. El Dorado, 10. Fuente de Oro, 11. Granada, 12. Guamal, 13. La Macarena, 14. La Uribe, 15. Lejanías, 16. Mapiripán, 17. Mesetas, 18. Puerto Concordia, 19. Puerto Gaitán, 20. Puerto Lleras, 21. Puerto López, 22. Puerto Rico, 23. Restrepo, 24. San Carlos de Guaroa, 25. San Juanito, 26. San Martín, 27.San Juan de Arama, 28. Villavicencio, 29. Vista Hermosa.

Municipalities in the Andean natural region and those under armed conflict (civil war) were excluded from the study. For 6 months (July-October 2009 and February-April 2010), trained staff (veterinarians and biologists) visited a total of 148 cattle farms, where the same number of people in charge of handling animals (producers, farmers, veterinarians, and animal scientists) were surveyed to determine which plants they recognized as toxic and queried as to their effects in animals. After the survey, animals in all farms were inspected to detect any signs associated with poisonous plant consumption. In areas where plants considered to be toxic were present, a taxonomic specimen was collected and sent for identification at the Colombian National Herbarium, where specimens were deposited with their respective voucher. To further support the information gathered in this study, only those plants perceived as toxic by at least two people were included. The data collected were analyzed using descriptive statistics. The identification rate was calculated taking into account the number of people identifying a particular plant as toxic in relation to the total number of surveys (148).

Results

A total of 19 plants were identified by more than 2 surveyed people as being poisonous to cattle. Each identified plant is listed in table 1, along with its common name, associated effects, and identification rate.

The plant most frequently identified as poisonous was *Enterolobium cyclocarpum* (figure 2), with an identification rate of 29.1 percent (49 respondents). The fruits of this plant are empirically recognized as a cause of photosensitization. Sudden death syndrome and photosensitivity were the more commonly reported manifestations of cattle intoxication. Other signs attributed to the consumption of these plants are frequent bone fractures, prostration, bloating, nervous signs, and muscular weakness.

The 19 plants are grouped in 9 recognized botanical families, with the largest number of plants being clustered under the family Apocynaceae (6), followed by Bignonaceae (3), Sapindaceae and Verbenaceae (2 plants each), and Cyperaceae, Heliconaceae, Fabaceae, Phytolaccaceae, and Solanaceae, each family with one plant.

Discussion

In order to consider a plant to be toxic for livestock, the poisoning must occur naturally, and the toxicosis must be reproduced under experimental conditions in the animal species involved (Tokarnia et al. 2000, Diaz 2010). Therefore, a careful assessment should be made before attributing a toxicological activity to a plant. Of the 19 plants empirically identified as being toxic to cattle in this study, toxicosis has only been successfully reproduced experimentally with *E. cyclocarpum* and *Petiveria alliacea*, which have been shown to produce the signs quoted by the surveyed population, i.e. photosensitization and muscle weakness, respectively (Núñez et al. 1983, Negrón et al. 1993).

Table 1. Plants identified as poisonous to cattle in the Departments of Meta and Casanare in Colombia by the surveyed population and the main clinical signs associated with their consumption.

Latin name	Family	Common name	(%)*	Main signs
Enterolobium cyclocarpum	Fabaceae	Caracaro	29.1	Photosensitization
Tabernaemontana siphilitica	Apocynaceae	Borrachero arbustivo, borrachero negro	12.8	Sudden death
Marsdenia rubro-fusca	Apocynaceae	Borrachero rojo	10.8	Nervous signs
Mesechites trifidus	Apocynaceae	Borrachero blanco	8.8	Sudden death
Mandevilla trianae	Apocynaceae	Borrachero blanco	8.8	Sudden death
Lantana cujabensis	Verbenaceae	Mermelada	6.1	Photosensitization
Tabernaemontana heterophylla	Apocynacae	Borrachero turma de gato, cojón	6.1	Sudden death
Stemmadenia grandiflora	Apocynaceae	Borrachero turma de perro, jazmín malabar	6.1	Sudden death
Solanum mammosum	Solanaceae	Lulo de perro, friega platos, pepito	4.1	Prostration
Heliconia latispatha	Heliconaceae	Heliconia	2.7	Bone weakness
Adenocalymma purpurascens	Bignonaceae	Barbasco cuatro filos	2.7	Nervous signs
Arrabidaea sceptrum	Bignonaceae	Bejuco mataganado	2.7	Sudden death
Mussatia prieurei	Bignonaceae	Bejuco mataganado	2.7	Sudden death
Serjania grandis	Sapindaceae	Rabo de iguana	2.7	Prostration
Lantana maxima	Verbenaceae	Mermelada	2.7	Photosensitization
Polygonum punctatum	Polygonaceae	Barbasco rojo	1.4	Nervous signs
Rhynchospora nervosa	Cyperaceae	Tote, estrella	1.4	Photosensitization
Paullinia alata	Sapindaceae	Bejuco camándula	1.4	Nervous signs
Petiveria alliacea	Phytolacaceae	Anamú	1.4	Muscle weakness

*Percentage of people who identified a plant as toxic in relation to the total number of people surveyed (148). A minimum of 2 respondents had to identify a plant as toxic for inclusion on this list.

Figure 2. Left, *Enterolobium cyclocarpum*, the most frequently identified poisonous plant affecting cattle, as reported by the surveyed population. Right, fruits of *E. cyclocarpum*, the part of the plant considered to be toxic.

According to the taxonomic classification, the plant family with the largest number of species was Apocynaceae (6), which collectively was the most recognized as toxic after *E. cyclocarpum* (Fabaceae). Among Apocynaceae species, *Stemmadenia grandiflora*, *Tabernaemontana siphilitica* (syn. *Bonafousia tetrastachya*), *Mandevilla* sp., and *Mesechites trifidus* have been previously reported by livestock producers to cause poisoning in cattle, with nervousness and death as the primary clinical signs associated with their consumption (Vargas et al. 1998, Velásquez et al. 2000). However, there are no studies describing the detailed clinical signs associated with their consumption either after natural or experimental poisoning.

Some Apocynaceae plants (*Asclepias curassavica*, *Nerium oleander*, *Thevetia peruviana*) are known to contain cardiac glycosides that can cause rapid death in humans and animals (Barbosa et al. 2008, Bandara et al. 2010), and their presence has been documented in Colombia (Diaz 2010, 2011). Similarly, the *Stemmadenia* and *Tabernaemontana* genera have been verified to contain indole alkaloids, which can be toxic (Van-Beek et al. 1984, Torrenegra et al. 1988, Upmanyu et al. 2009). It is therefore necessary to investigate the identity of the metabolites present in the Apocynaceae species reported here as well as to conduct studies to determine the toxic effects of these plants in cattle.

In the present study, *Arrabidaea sceptrum* was recognized as a cause of sudden death. Although there are no scientific studies reporting this particular species to be toxic, other species of the genus, such as *A. bilabiata* and *A. japurensis*, have been noted to have the same effect on cattle (Tokarnia et al. 2002). Additionally, *A. bilabiata* has been reported to contain monofluoracetic acid (Krebs et al. 1994), which is a highly toxic substance that can block the citric acid cycle and cause rapid death (Diaz 2010). It is possible that *A. sceptrum* contains the same toxic compound as *A. bilabiata*, but this hypothesis must be tested.

Lantana maxima and *L. cujabensis* are species without reports of toxicological implications; however, another species of this genus, *L. camara*,

has been found to contain lantadenes, compounds that cause liver damage. Lantadenes affect the biliary excretion of photoactive compounds and cause photosensitization (Sharma et al. 2007), an effect that has been attributed to the *Lantana* spp. reported here.

Paullinia alata belongs to a genus of plants known for their content of methylxanthines (e.g. caffeine, theobromine, and theophylline) (Carlson and Thompson 1998). Other members of this genus include *Paullinia cupana* (guaraná), which is used in the production of energy drinks (Carlini 2003). It is possible that the nervous signs attributed by the surveyed workers to *P. alata* are the result of CNS stimulation caused by these compounds.

Based on the ethnobotanical use of some of these plants, several authors have identified a pharmacological activity associated with them. Specifically, we refer to *Solanum mammosum*, which is used in the treatment of respiratory disease due to its antimicrobial activity (Caceres et al. 1991); *Heliconia latispatha*, which is effective in the treatment of snake bites due to its proven haemolytic effect on *Bothrops asper* venom (Pereañez et al. 2008); and *Arrabideae sceptrum*, which has anti-inflammatory as well as antiviral activity (Brandao et al. 2010). Plant toxins can often be used as effective treatments for the same diseases (Molyneux et al. 2007). For example, *Polygonum punctatum*, recognized as toxic in this study, has antifungal activity that has been attributed to polygodial, a sesquiterpene aldehyde found in this plant (Almeida-Alves et al. 2001). Toxic plants may also be a rich source of new drug compounds, and therefore the general knowledge provided by the livestock or ethnobotany community is very useful for designing future studies related to the discovery of new pharmacologically interesting molecules. On the other hand, some of the plants cited here have not been reported to have any physiological activity (*Marsdenia rubrofusca*, *Adenocalymma purpurascens*, and *Serjania grandis*), which supports the need to conduct the appropriate phytochemical studies.

Regarding the signs and lesions associated with the plants reported in this study, sudden death and photosensitization were most frequently reported. Sudden death is a clinically inexplicable death that occurs rapidly (within 12-24 hours) during normal activity in apparently healthy animals with no history of significant disease (Bradford 1996). Nitrates, cyanogenic glycosides, cardiac glycosides, monofluoracetic acid, and some alkaloids are possible toxins that can cause sudden death (Tokarnia et al. 2000, Knight and Walter 2001). It is important to assess the potential presence of these toxins in the plants reported to cause sudden death.

Photosensitization was frequently reported by the surveyed people, and its occurrence was verified by field researchers in 85 percent (126) of the farms visited (figure 3). Plants reported to cause this condition were *Enterolobium cyclocarpum*, *Rhynchospora nervosa*, and *Lantana* spp. However, it is important to note that *Brachiaria* spp. (*B. decumbens*, *B. brizantha*, *B. humidicola*, *B. dyctioneura*, and a hybrid known as Mulato) are found abundantly in the studied area. This forage grass is fed to cattle as well as provided on many cultivated pastures. *Brachiaria* spp. are known to cause photosensitivity secondary to liver damage (Diaz 2011). In this study, 93 percent of the farms had *Brachiaria* pastures, which often contain steroidal saponins that can cause liver damage leading to photosensitization (Riet-Correa et al. 2011). It is essential to investigate which is the cause of the photosensitization in the cattle raised in this area of the Colombian eastern plains and to identify the relative contributions of *Enterolobium cyclocarpum*, *Rhynchospora nervosa*, *Lantana* spp., and *Brachiaria* spp. to cattle photosensitization.

Summary

Studies on plants toxic to animals are scarce in Colombia, even though this country has the second highest plant biodiversity in the world. A recent publication reports about 150 species of potentially toxic plants that comprise almost 100 genera and 34 botanical families (Diaz 2010). However, there is still a large number of species that remain to be studied and which can contribute to this problem. The present study adds to knowledge about toxic flora of the eastern zone of Colombia, where a high percentage of the cattle population in Colombia is located. The identification of the toxic plants prevailing in each region will help to decrease their economic impact on livestock farms. It is essential to conduct studies to confirm the ethnobotanical information associated with these plants using appropriate experimental models (ruminants) and to determine the identity of potentially toxic compounds. It should also be noted that these plants may contain compounds of pharmacological interest with potential applications in the treatment of human diseases.

Figure 3. Bovine (Nelore breed) with evidence of photosensitization.

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Effects of *Brachiaria brizantha* Hay Containing a Steroidal Saponin in Lambs

Flavia G. Lima[1]*, Carolina S. Ribeiro[1], Diogo D. F. Andrade[1], Gustavo L. Costa[1], Hugo C. M. Pires[1], Victor Y. Guimaraes[1], Mitsue Haraguchi[2], and Maria Clorinda S. Fioravanti[1]

[1]Federal University of Goiás, Goiânia, GO, Brazil
[2]Biological Institute, São Paulo, SP, Brazil

*Corresponding author: Flavia G. Lima, flaviamedvet@yahoo.com.br

Abstract

Several species of *Brachiaria* are important forages in tropical regions of Brazil and elsewhere worldwide. Some species of *Brachiaria* have been reported to cause hepatogenous photosensitization in ruminants. Initially, the disease was attributed to *Pithomyces chartarum* fungus, but recent studies suggest that the steroidal saponins present in the grasses have toxic principles responsible for the photosensitization. The objective of this study was to evaluate hepatic function and the performance of lambs fed with *B. brizantha* hay or sugar cane (*Saccharum officinarum* L.), using clinical examinations, laboratory tests, and macro and microscopic analysis of the liver. Twelve Saint Ines lambs were used. The animals were divided into two experimental groups: group hay (six lambs fed with *B. brizantha* hay, plus a concentrate feed) and group sugar cane (six lambs fed with roughage from sugar cane with added concentrate feed). The hay and sugar cane used to feed the lambs did not contain *Pithomyces chartarum* spores. The *B. brizantha* hay contained 0.86% protodioscin, and the animals received a daily protodioscin dose of 200 mg/kg BW. The clinical examination occurred every 7 days, the laboratory tests were done every 14 days, and the animals were weighed every 21 days. At the end of 93 days of feeding, the lambs were slaughtered, the macroscopic analysis of the organs was carried out, and the liver fragments were collected for histological analysis. The lambs were clinically healthy during the whole period, except at the beginning of the experiment when some animals had pneumonia. The only biochemistry alteration suggestive of hepatic damage was an increase ($P < 0.05$) of the GGT values in both groups. No animal fed with *B. brizantha* hay showed any macroscopic alterations in the liver. Histological analysis of the liver revealed preserved hepatocytes and the presence of light multifocal infiltration of mononuclear inflammatory cells in the hepatic parenchyma and also in the portal space, indicating mild cholangitis in both groups. Degeneration changes suggestive of hepatic steatosis were observed in four animals fed with sugar cane. Feeding lambs with *B. brizantha* hay promoted similar performance as feeding animals with sugar cane. We conclude that feeding *B. brizantha* hay containing the concentration of steroidal saponins used in this study to lambs does not cause toxicity.

Keywords: *Brachiaria brizantha*, cholangitis, gamma glutamyltransferase, GGT, hepatogenous photosensitization, protodioscin, saponin

Introduction

In recent years the sheep industry in Brazil has grown significantly compared with the number of cattle, probably due to the numerous advantages offered such as the possibility to produce animals in

small holdings, lower individual animal forage intake, ease of handling, and the production of quality products such as milk, meat, and leather (Oliveira 2001).

Livestock losses can be attributed to the decrease in performance as a result of infections, poisoning, genetic and nutritional diseases, or deaths. In the Brazilian cattle industry, as well as in many other countries, a significant cause of loss is by the ingestion of poisonous plants. It is estimated that 9 to 16 percent of cattle deaths in Brazil is from poisonous plants (Assis et al. 2010).

Weight loss or the inability to gain weight in livestock may be due to clinical and subclinical diseases that affect the animal's health, which can sometimes lead to death. Among these are diseases that cause liver damage, such as hepatogenous photosensitivity, which may occur from the toxin produced by *Pithomyces chartarum* fungus (Di Menna et al. 2009) or may be caused by steroidal saponins often found in forage from the genus *Brachiaria*. Saponins have been associated with the deposition of crystalloid material in the biliary system (Brum et al. 2007).

At present, the progression from ingestion of steroidal saponins in *Brachiaria* spp. to pathogenesis and hepatic lesions in ruminants is unclear. The objective of this research was to conduct a controlled study by feeding a known amount of the steroidal saponin protodioscin in *Brachiaria brizantha* hay to lambs and to evaluate clinical signs of toxicity in conjunction with macro and histological analysis of potential liver lesions.

Materials and Methods

Twelve 104-day-old Santa Ines lambs were used. The animals were divided into two groups: the *Brachiaria* Group (BG)—six lambs fed with hay from *Brachiaria brizantha* Hochst ex A. Rich.) Stapf. cv. Marandu and the control Sugar Cane Group (SCG)—six lambs fed with roughage made from ground sugar cane (*Saccharum officinarum L.*) stems and leaves, *in natura*.

The experiment was conducted over 93 days at the experimental facility for ruminants at the Veterinary School at the Federal University of Goiás, Brazil, located in the city of Goiânia, from June to September 2008. This animal study was conducted under veterinary supervision under animal ethics protocols established by the Brazilian Society of Laboratory Animal Science (SBCAL, formerly Brazilian College of Animal Experimentation/ COBEA).

Concentrate feed (finely ground corn, soybean, and cottonseed) was used to balance the nutritional requirements of the two groups of animals and to minimize the nutritional differences between the two diets. The amount of total feed provided was limited to 4 percent of body weight. The proportion of roughage and concentrate was 62:38, and this amount was recalculated after every weighing of the animals.

For saponin analysis, samples were collected of fresh plant, freshly dried hay, and hay after storage for 3 months. Samples were dried in a forced air oven at 65°C for 72 hours, ground to pass a 2 mm screen in a Wiley mill, and stored in airtight bags until analysis. Saponin extraction and analysis were conducted at the Biological Institute, São Paulo, Brazil. The extraction was made in duplicate. Each sample (1 g) was extracted 3 times in 50% acetonitrile and water (v/v), using volumes of 8 mL, 5 mL, and 3 mL under agitation with a sonic agitator for 30 min, 30 min, and 20 min, respectively. Next the extracts were transferred to a test tube and centrifuged for 30 minutes at 5,000 rpm at 13ºC. The extracted solution was completed to 10 mL for analysis using HPLC/ELSD (high-performance liquid chromatograph/evaporative light scattering).

Saponin was quantified using a modification of the technique of Ganzera et al. (2001). The analysis was done with a Shimadzu HPLC model LC-10AD coupled to the detector; the evaporative light scattering from the Shimadzu model ELSD-LTs was used for quantitative analysis of protodioscin. The column used was a Shim-Pack CLC-ODS (4.6 x150 mm, 5.0 µm), using a gradient system with acetonitrile (B)/water (A), starting at 20% B, 5 min; 35% B, 12.5 min; and 20% B, 5 min; flow 1 mL/min, injected with 10 µL, using a 10 µL loop.

The only saponin detected in *B. brizantha* was protodioscin (Brum et al. 2009). The BG received 62 percent of their daily ration from *B. brizantha* hay at 4 percent of their body weight, which corresponded to a daily average protodioscin dose of 200 mg/kg BW for the 93-day experiment. There were no detectable levels of protodioscin in sugar cane.

The animals involved in this research had not been previously fed with *Brachiaria brizantha* forage or with sugar cane; neither the hay nor the sugar cane contained *Pithomyces chartarum* spores.

Animals were weighed every 21 days after an overnight fast. After slaughter, the carcass was weighed to determine the yield. The liver was weighed separately. Clinical evaluations were made weekly according to the protocol adapted from Rosenberger (1983).

Blood was sampled every 14 days using jugular venipuncture beginning on day zero to day 70 except for the final collection, which occurred on day 93 at the end of the study. Samples were handled by standard methods for serum and whole blood. The tests performed were hemogram, fibrinogen, aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), total, conjugated and non-conjugated bilirubin, total protein, urea, creatinine, total cholesterol, glucose, and protein separation by electrophoresis. The results of red blood cells (RBC) were compared with those of Ferreira (2002), white blood cells (WBC) with the values described by Jain (1993) for sheep, and biochemistry and electrophoresis, respectively, with Kaneko et al. (2008) and Green et al. (1982).

Macroscopic examination of animals during slaughter was done by evaluating the lungs, lymph nodes, liver, gallbladder, bladder, kidney, pre-stomach compartments (rumen, reticulum, and omasum), abomasum, and intestines (small and large). Liver samples were collected (left lobe) for microscopic analysis.

The liver samples were fixed in 10% buffered formalin and, after 48 hours, stored in 70% ethanol. Subsequently, they were processed according to routine laboratory standards and stained with hematoxylin and eosin. We conducted a descriptive analysis of the lesions observed in the parenchyma and portal areas.

Statistical analysis between the two treatment groups for various blood variables was done using the non-parametric Mann-Whitney U test at significant levels of 5 percent.

Results

The level of protodioscin in the fresh plant was 1.87 percent; however, both the freshly-made *Brachiaria* hay and hay after storage had protodioscin concentrations of 0.86 percent.

The lambs exhibited normal behavior and food intake throughout the experiment, except for a reduction in food intake near the end of the study when the rainy season began. Three lambs in the SCG and one in the BG had pneumonia at the beginning of the experiment and were immediately treated with florfenicol and flunixin meglumine. No animal showed any further signs of any other diseases.

The clinical evaluations did not reveal any differences between treated animals, nor were there differences in RBC, hemoglobin, hematocrit, WBC, or fibrinogen (table 1). The initial and final weight of the BG lambs was 28 kg (±3.1) and 35.5 kg (±5.1), respectively, whereas the initial weight and final weight for the SCG lambs was 27 kg (±2.4) and 37 kg (±3.7), respectively ($P > 0.05$). Carcass yield (dressing %) was similar between the groups (BG = 46.14±6.55% and SCG = 46.17±1.41%), as was the weight of the liver (BG = 0.55±0.12 kg and SCG = 0.57±0.11 kg).The macroscopic evaluations of the BG animals showed that the liver, gallbladder, and bile were normal; one animal had pale spots on the cortical surface of the kidneys and one animal showed an area of rumen papillae atrophy and hyperemia. In the SCG, one animal showed an increased liver lobular pattern and another showed a pale liver; one animal showed lesions suggesting interstitial nephritis; and another animal showed focal areas of ulcers and scars 1 cm in diameter in the ruminal mucosa. The gallbladder and bile of all animals were within normal limits.

The evaluation of lungs in both groups proved to be consistent with the clinical evaluation. The lungs of animals suffering from pneumonia had areas of consolidation, hyperemia, emphysema, and in some cases, abscesses.

Histological evaluation of both groups revealed preserved hepatocytes and the presence of light multifocal mononuclear cell infiltration in the liver parenchyma and portal space. In the SCG, four animals were found to have micro and macrovacuolar degeneration suggesting hepatic steatosis, restricted to zones 1 and 2 of the hepatic acinus. None of the groups showed the presence of foamy macrophages, bile pigments, or crystals. The presence of mononuclear infiltrate in the portal space, usually around the bile ducts, suggested mild cholangitis.

Discussion

The naive lambs never exposed to *Brachiaria* forage were chosen because these animals were considered to be most susceptible to poisoning (Riet-Correa and Mendez 2007). However, lambs in the BG fed with *B. brizantha* hay showed no clinical signs of poisoning, demonstrating that, under these experimental conditions, the protodioscin dose of 200mg/kg BW was not toxic. In another study, forage levels of protodioscin above 0.3 percent were enough to poison sheep of the same breed (Castro et al. 2011). Other studies report that, in spite of an increase in some liver enzymes from 42.8 to 100 percent in sheep experimentally intoxicated by *Brachiaria* spp., these same animals did not show

Table 1. Average and standard deviations for AST, ALP, GGT, conjugated bilirubin (CB), unconjugated bilirubin (UCB), total bilirubin (TB), glucose, total cholesterol, total protein, albumin, alpha 1, alpha 2, beta and gamma globulins, albumin/globulin (A/G), urea and creatinine, for *Brachiaria*-fed (BG) lambs and lambs fed sugar cane (SCG) over 93 days

Biochemistry Test	Group	A/SD	D0	D14	D28	D42	D56	D70	D93	Ref
AST U/L	BG	A	131.00*	85.00	99.17	100.00	93.00	102.50*	98.17	60-280[1]
		SD	16.69	15.17	7.36	24.51	6.93	18.45	3.76	
	SCG	A	101.33	85.00	94.83	106.00	88.67	84.17	96.67	
		SD	14.76	17.33	21.70	32.22	8.85	4.22	13.68	
ALP U/L	BG	A	130.17	78.67	62.17	60.67	137.00	165.67	156.50	68-387[1]
		SD	46.58	14.95	19.08	34.15	62.50	64.08	77.17	
	SCG	A	126.67	85.83	57.50	71.50	194.17	176.67	147.00	
		SD	42.26	37.10	21.04	42.28	89.28	29.86	26.45	
GGT U/L	BG	A	52.65	60.33	48.85	29.08*	52.18	49.97	81.85	20-52[1]
		SD	14.37	22.37	13.78	6.65	9.77	13.41	26.01	
	SCG	A	64.54	69.07	57.27	48.57	54.28	51.45	72.68	
		SD	26.68	18.17	12.80	7.24	14.70	13.46	21.17	
CB µmol/L	BG	A	0.74	0.83	0.91	1.20	0.88	1.71	1.77	0.0-4.61[1]
		SD	0.21	0.41	0.55	0.96	0.74	1.70	1.92	
	SCG	A	0.68	1.08	1.17	0.74	0.74	1.11	1.08	
		SD	0.11	0.92	1.04	0.34	0.26	1.24	0.86	
UCB µmol/L	BG	A	4.67	3.88	5.73	2.57*	3.68	2.65	3.62	0.0-2.05[1]
		SD	1.88	2.36	2.42	1.04	0.95	1.92	0.95	
	SCG	A	5.02	4.53	4.87	4.59	4.45	3.59	3.65	
		SD	1.29	2.08	1.66	0.91	0.47	1.35	1.81	
TB µmol/L	BG	A	5.42	4.70	6.64	3.76*	4.56	4.36	5.39	1.71-8.55[1]
		SD	2.04	1.96	2.40	0.96	0.85	1.68	1.39	
	SCG	A	5.70	5.61	6.04	5.33	5.19	4.70	4.73	
		SD	1.31	1.81	1.38	0.90	0.35	0.89	1.29	
Glucose mmol/L	BG	A	3.90	3.60	3.98	2.79	3.18	3.53	3.88	2.78-4.44[1]
		SD	0.53	0.15	0.43	1.61	0.45	0.38	0.60	
	SCG	A	3.58	3.87	4.23	4.73	3.31	3.14	3.92	
		SD	1.94	0.36	0.30	1.29	0.13	0.35	0.81	
Cholesterol (total) mmol/L	BG	A	1.71	1.23*	1.19	1.25*	1.24*	1.50*	1.09*	1.35-1.97[1]
		SD	0.31	0.09	0.59	0.25	0.17	0.25	0.07	
	SCG	A	1.32	0.88	1.13	0.60	0.71	0.67	0.63	
		SD	0.30	0.31	0.34	0.23	0.22	0.44	0.12	
Total Protein g/L	BG	A	70.75*	80.67	81.50	61.50	72.00	71.83*	78.00*	60-79[1]
		SD	2.36	6.19	16.67	8.69	10.55	6.46	10.71	
	SCG	A	62.67	76.17	66.50	54.17	65.00	65.00	63.00	
		SD	4.61	2.56	3.94	4.71	5.48	2.19	6.32	
Albumin g/L	BG	A	37.00	40.00	41.00	27.00	39.00	39.00	44.00	24-30[1]
		SD	3.00	3.00	8.00	2.00	6.00	3.00	7.00	
	SCG	A	34.00	39.00	33.00	29.00	36.00	37.00	36.00	
		SD	2.00	4.00	4.00	4.00	3.00	3.00	3.00	
Alpha 1 g/L	BG	A	8.00	8.00	8.00	5.00	5.00	5.00	6.00	2.7-4.1[2]
		SD	1.00	1.00	2.00	1.00	1.00	1.00	1.00	
	SCG	A	6.00	7.00	6.00	4.00	5.00	5.00	4.00	
		SD	1.00	1.00	1.00	0.00	1.00	0.00	1.00	
Alpha 2 g/L	BG	A	10.00	11.00	10.00	9.00	8.00	9.00	9.00	4.9-5.8[2]
		SD	1.00	1.00	2.00	1.00	1.00	2.00	1.00	
	SCG	A	9.00	10.00	10.00	7.00	8.00	8.00	8.00	
		SD	0.00	1.00	2.00	1.00	1.00	1.00	1.00	
Beta g/L	BG	A	5.00	5.00	4.00	4.00	3.00	3.00	3.00	4.0-14.0[1]
		SD	1.00	1.00	1.00	2.00	1.00	0.00	1.00	
	SCG	A	3.00	4.00	4.00	2.00	3.00	3.00	3.00	
		SD	3.00	1.00	1.00	1.00	0.00	1.00	1.00	

Biochemistry Test	Group	A/SD	D0	D14	D28	D42	D56	D70	D93	Ref
Gamma g/L	BG	A	12.00	17.00	18.00	16.00	17.00	16.00	16.00	14.4-20.7[2]
		SD	2.00	4.00	6.00	6.00	4.00	3.00	3.00	
	SCG	A	12.00	15.00	15.00	12.00	14.00	12.00	12.00	
		SD	4.00	3.00	3.00	2.00	2.00	2.00	2.00	
A/G	BG	A	11.00	10.00	10.00	8.00	12.00	12.00	13.00	4.2-7.6[1]
		SD	2.00	1.00	1.00	2.00	1.00	1.00	1.00	
	SCG	A	12.00	11.00	10.00	12.00	12.00	14.00	14.00	
		SD	2.00	2.00	3.00	3.00	1.00	2.00	0.00	
Urea mmol/L	BG	A	7.75	6.63	8.36	6.90*	7.19	7.35	9.15	2.86-7.14[1]
		SD	1.13	1.27	3.76	1.75	1.01	2.47	3.28	
	SCG	A	7.49	5.88	6.96	10.81	7.55	10.20	8.77	
		SD	1.59	1.94	2.11	1.73	1.08	2.76	2.74	
Creatinine µmol/L	BG	A	82.51	91.35	86.93	71.90	101.66	81.03	76.61	106-168[1]
		SD	6.04	12.08	6.65	33.95	4.84	6.65	16.46	
	SCG	A	81.77	85.45	82.51	67.77	95.77	75.14	69.25	
		SD	9.17	9.13	12.08	7.22	11.75	18.33	8.69	

A/SD = average and standard deviation. Ref = Reference. D=day of the study.
[1] Kaneko et al. (2008)
[2] Green et al. (1982)
*Statistical analysis was done using the Mann-Whitney U Test.
Asterisks indicate that the treatment groups differ at $P < 0.05$ during the same period.

clinical signs of hepatogenous photosensitization (Cruz et al. 2001, Saturnino et al. 2010, Castro et al. 2011).

In this study, there were no differences between the *Brachiaria*-fed lambs and the control lambs in terms of body weight, nor were there differences for carcass yield and liver weight. In contrast, other work has shown that sheep given steroidal saponins in *Panicum miliaceum* forage, while maintaining a normal appetite, did lose some body weight, and this loss progressed to cachexia (Badiei et al. 2009).

Consistent with the pneumonia that we observed at the beginning of the study, the initial leucocyte counting and electrophoresis revealed an acute inflammatory process that gradually decreased until it was chronic at the end of the experiment. The treatments did not influence the inflammatory response because both groups showed the same trends. Saturnino et al. (2010) evaluated the toxicity to Santa Ines sheep from *B. decumbens* forage in a feedlot and also reported cases of pneumonia.

In this study, AST values were within physiological limits for both groups. When studying hepatogenous photosensitization in sheep fed with *Brachiaria* spp., Castro et al. (2011), Saturnino et al. (2010), Mendonça et al. (2008), and Brum et al. (2007) also found values of AST very close to the reference limit. AST is an enzyme that has two isoenzymes, one mitochondrial and one cytoplasmic. Together they determine the integrity of the hepatocyte, but AST is only elevated in acute liver injury, immediately returning to the reference limits (Kaneko et al. 2008).

The increased values of serum GGT activity in both groups suggested lesions in the biliary epithelium, which were confirmed in the histological analysis. We noted the presence of mononuclear cell infiltration in portal spaces that featured mild cholangitis. The elevation of GGT has been a recurrent finding in sheep poisoned by *Brachiaria* spp. in the absence of *P. chartarum* spores (Lemos et al. 1996, Brum et al. 2007, Mendonça et al. 2008, Albernaz et al. 2010, Assumaidaee et al. 2010, Saturnino et al. 2010, Castro et al. 2011). Interestingly, Cruz et al. (2011) administered fractionated extracts of *B. decumbens* and observed no changes in serum GGT activity and no clinical signs; however, their animals showed experimentally induced cholangiohepatopathy. According to Blackshaw (1978) and Pearson (1993), GGT shows sensitivity and specificity and almost always is high in cases of chronic liver disease. Increased GGT is directly related to the death of sheep poisoned by *B. decumbens* and can be used as a parameter to predict the onset of clinical signs and death of sheep that ingest this forage (Saturnino et al. 2010).

The high levels of unconjugated bilirubin and albumin reported here were not attributed to *Brachiaria* forage because these variables were elevated in both groups. The primary cause of hyperalbuminemia is dehydration (Russell and

Roussel 2007), but the animals showed no signs of dehydration.

The serum urea values were above the reference limits in most periods in both groups. The high levels of urea in this study can be attributed to the diets fed to both groups. The possibility of elevated levels of urea because of renal failure was discarded by analyzing creatinine, which was at or below reference levels. Castro et al. (2011), evaluating the same breed of sheep fed with *B. brizantha* and *B. decumbens*, found increased levels of urea and creatinine.

In several studies of sheep intoxicated by *Brachiaria* spp., histological findings in the liver have shown multifocal areas of pallor, hepatomegaly, printing on the surface of the ribs, hepatic lobular pattern, jaundiced and distended gallbladder, dark colored bile, and increased density (Lemos et al. 1996, Cruz et al. 2001, Driemeier et al. 2002, Brum et al. 2007, Mendonça et al. 2008, Albernaz et al. 2010, Assumaidaee et al. 2010, Saturnino et al. 2010, Castro et al. 2011). Even so, none of these lesions were found in the BG animals in our study, and Castro et al. (2011) also reported no histopathological changes in sheep poisoned by *Brachiaria* spp.

In the present study, the presence of multifocal mononuclear infiltrates in the parenchyma and portal areas (mild cholangitis) showed that the structure of hepatocytes and bile duct were preserved. Several studies have described the presence of mononuclear infiltrates around the portal space but were followed by other findings characteristic of intoxication, such as infiltrates of foamy macrophages and lymphocytes in the parenchyma and portal spaces, bile duct proliferation accompanied by epithelial degeneration, necrosis and hyperplasia, multifocal areas of cholangitis with accumulation of bile pigments, presence of crystals within the bile ducts and macrophages, diffuse swelling and vacuolization of hepatocytes (Lemos et al. 1996, Cruz et al. 2001, Driemeier et al. 2002, Brum et al. 2007, Mendonça et al. 2008, Albernaz et al. 2010, Assumaidaee et al. 2010, Saturnino et al. 2010, Castro et al. 2011).

Degenerative changes only present in the SCG can be attributed to the high availability of carbohydrates from the sugar cane at amounts substantially higher than in *Brachiaria* hay, which could have caused a lipid overload in the liver causing hepatic steatosis (Santos 1986). Future studies should evaluate the effect of sugar cane on liver function in sheep.

We conclude that lambs fed with *Brachiaria brizantha* hay containing 0.86 percent of protodioscin showed no decrease in performance and did not show clinical signs of poisoning. Other comparable studies will need to be conducted using known types and concentrations of steroidal saponins to clarify the etiology of hepatogenous photosensitization in ruminants consuming *Brachiaria* spp. forage.

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The Resolution of Rayless Goldenrod (*Isocoma pluriflora*) Poisoning in Goats

Bryan L. Stegelmeier[1*], T. Zane Davis[1], Benedict T. Green[1], Stephen T. Lee[1], and Jeffery O. Hall[2]

[1]USDA-ARS Poisonous Plant Research Laboratory, Logan, UT
[2]Utah Veterinary Diagnostic Laboratory, Logan, UT

*Corresponding author: Bryan L. Stegelmeier, bryan.stegelmeier@ars.usda.gov

Abstract

Rayless goldenrod (*Isocoma pluriflora*) occasionally poisons livestock causing myocardial and skeletal muscle degeneration and necrosis. The objectives of this study were to describe the resolution of the clinical and pathological changes of rayless goldenrod poisoning in goats. Eight goats were gavaged for 7 days with ground rayless goldenrod to obtain benzofuran ketone dosages of 40 mg/kg BW/day. After treatment, three goats were euthanized and the other goats were allowed to recover for 10 days and 2, 4, 8, and 16 weeks. After 6 days of treatment, all the treated animals were reluctant to move, stood with an erect stance, and became exercise intolerant. Serum enzymes such as AST, ALT, LDH, and CK had elevated activities indicative of muscle damage. Animals quickly recovered and showed few clinical signs at 2 weeks post treatment. Histologically, poisoned goats developed severe skeletal myodegeneration and necrosis characterized by myocyte swelling and hypereosinophilia, clumping and aggregation of myofibers, and myocyte disruption with extensive perimysial edema and inflammation. This degeneration and necrosis persisted with increased inflammation in the goats that were euthanized at 10 days and 2 weeks post treatment. In animals that were allowed to recover for 4 and 8 weeks, there was progressively less degeneration, necrosis, and inflammation with more edema, regeneration, and fibrosis. After 16 weeks there was edema and mild fibrosis. These findings indicate that rayless goldenrod poisoning causes skeletal muscle necrosis that continues to resolve 3 months after exposure. Though the remaining lesions are minimal, complete resolution could take many additional months.

Keywords: goats, *Isocoma pluriflora*, rayless goldenrod

Introduction

Rayless goldenrod or jimmyweed [*Isocoma pluriflora* (Torr. & A. Gray) Greene (Asteraceae) previously *Isocoma wrightii* (A. Gray) Rydb and *Happlopappus heterophyllus* (A. Gray) S.F. Blake] is found and sporadically poisons livestock in Arizona, Colorado, New Mexico, and Texas (figure 1). All species are probably susceptible, but poisoning has only been reported in horses, cattle, pigs, sheep, goats, and humans (Burrows and Tyrl 2001). First described as "alkali disease" in the early 1900s, rayless goldenrod poisoning was erroneously associated with drinking saline or alkaline water (Marsh and Roe 1921, Marsh 1926). Clinically, poisoned livestock are depressed, anorexic, and reluctant to move. Some poisoned animals may develop tachypnea and tachycardia with ascites and hydrothorax. Ultimately, most develop violent trembling when forced to move. Both the skeletal muscle and myocardial lesions are due to diffuse and severe muscle degeneration and necrosis with subsequent fibrosis and atrophy (Stegelmeier et al. 2010). Poisoning by

Figure 1. Rayless goldenrod (*Isocoma pluriflora*) from near Pecos, TX. It is an erect, 30-to-120-cm-tall, bushy perennial that arises from a woody rootstalk. It is unbranched or sparsely branched with alternate, linear leaves. It has between 7 and 15 yellow flowers that form heads with flat clusters of the tips of the stems. It commonly grows in alkaline and gypsic soils in riparian zones along river valleys, drainage areas, or dry plains in southern Colorado, Texas, New Mexico, and Arizona.

rayless goldenrod usually occurs during fall and winter, when frosts may make rayless more palatable, when other forages have been depleted, or when snow makes alternative forages less accessible.

In 1930 tremetol, a straw yellow thick oil, was isolated from rayless goldenrod (Couch 1927, 1929, 1930). Recent work demonstrated that tremetol from rayless goldenrod is a mixture of benzofuran ketones including tremetone, dehydrotremetone, and 3-oxyangeloyl-tremetone (Zalkow et al. 1962, Lee et al. 2009). As these toxins or one of their metabolites may be excreted in milk, secondary poisoning of nursing neonates with reduced or no apparent maternal toxicity is of concern, and clinical cases have been reported (Burrows and Tyrl 2001).

The objectives of this study are to characterize and describe the resolution of rayless goldenrod and correlate these lesions with the clinical and biochemical-induced lesions to better predict the permanent sequelae of sublethal poisoning.

Materials and Methods

Plant Material

Rayless goldenrod was collected in Pecos City, TX (06˚42.656' N / 34˚74.847' E). The plant was taxonomically identified as rayless goldenrod (*Isocoma pluriflora*; Intermountain Herbarium at Utah State University, Logan, UT vouchers 250012 and 250014). The plant was air-dried, and 1 day prior to the beginning of the study, it was ground to pass through a 2.38 mm screen and thoroughly mixed to insure homogeneity. Concentrations of the benzofuran ketone compounds (tremetone, dehydrotremetone, and 3-oxyangeloyl-tremetone) were determined using previously described techniques (Lee et al. 2009). The relative proportions of benzofuran ketones in the dosed material were 12.5% tremetone, 32% dehydrotremetone, and 55.5% 3-oxyangeloyl-tremetone.

Animals

Eight yearling female Spanish goats weighing about 30 kg were trained to lead and to run on a treadmill for 3 weeks before the start of the study. The day before the initial dosing, all animals were weighed, bled by jugular venipuncture, and exercised on a treadmill while their electrocardiograms (ECGs) were monitored and recorded. For 7 consecutive days the goats were dosed intraruminally using an oral speculum and a 1-cm-diameter gastric tube with ground rayless goldenrod that contained 0.21% benzofuran ketones to obtain dosages of 40 mg benzofuran ketones/kg BW/day. Three control goats from the same herd were also trained and given ground alfalfa/grass hay using the same dosing method. Water and alfalfa hay were available ad libitum to all animals throughout the study. Throughout the study, all animals were monitored and exercised, and serum was collected weekly. After 7 days of dosing, the goats were randomized, and three treatment goats and the three control goats were euthanized and necropsied. The remaining animals were necropsied on day 10 and weeks 2, 4, 8, and 16 after treatment. At necropsy, samples of left lateral retro-ocular, tongue, masseter, superficial pectoral, triceps, intercostal, longissimus dorsi, semitendinosus, diaphragm, biceps femoris, biceps brachii, quadriceps femoris, gluteus medius, psoas major, adductor ,and semimembranosus skeletal muscles were collected, attached to wooden tongue depressors, and fixed in 10% neutral buffered formalin. The heart was opened, cleaned with water, examined, fixed intact, and later sectioned to examine portions of right atrium, right papillary muscle, right ventricular wall, septum, left atrium, left papillary muscle, and left ventricular wall. Other tissues including brain, spinal cord, lung, liver, kidneys, adrenal gland, urinary bladder, thyroid gland, lymph node, esophagus, rumen, omasum, abomasum, duodenum, pancreas, jejunum, ileum, cecum, and colon were collected, fixed, and prepared for microscopic examination. Tissues were

processed, sectioned, and stained using standard histologic techniques. To better demonstrate proliferation of fibrous connective tissue, select skeletal muscle slides were stained with Masson's trichrome stain.

Serum and ECG Analyses

Serum biochemistry and electrolyte analyses were performed using standard techniques with a Hitachi 7180 biochemistry analyzer (Hitachi High Technologies Inc., Pleasanton, CA). Reagents and methodology recommended by the manufacturers were used. Exercise tolerance and ECGs were done as previously reported (Stegelmeier et al. 2010).

Statistical Analyses

This study had limited sample sizes so our conclusions must be interpreted with caution. Our primary interest was in comparative histology. However, when *n* was larger than 2, the weekly serum biochemical data were analyzed as repeated measures over the multi-day experiment. Animals were a random factor in the mixed linear model analysis using the procedures of SAS (SAS Inst. Inc., Cary, NC; Version 9.1 for Windows). The primary dependent variable in the study was recovery time. The variance-covariance matrix was chosen by an interactive process wherein the best fit was based on the Schwarz Bayesian criterion. The unstructured and compound symmetry covariance models were often the best fitting structures. Treatment differences were separated using predicted difference (PDIFF option in SAS) for significant interactions ($P < 0.05$) in the model.

Results

After 5 days of dosing, the treated goats became reluctant to move and preferred to remain recumbent. When forced to stand, they would stand with post-like, straight legs, their backs flexed in a humped up position, and their tails flexed vertically. On days 6 and 7, many of the large appendicular muscles were swollen and firm. When standing, poisoned animals would tremble, quickly fatigue, and lie down. Details of the exercise physiology and ECG findings similar to those seen during the rayless goldenrod treatment were previously reported in detail (Stegelmeier et al. 2010). Briefly, we found that poisoned animals had an increased heart rate, and prolonged times were required for exercise-induced tachycardia to resolve (increased recovery times after exercise). No other ECG abnormalities were identified.

Immediately after treatment, the recovery goats continued to be reluctant to rise and move. When forced to walk on the treadmill, they quickly fatigued and refused to walk. These signs were of similar intensity for the first recovery week; however, they progressively improved during the second week. Improvement was slower for the remaining portion of the entire recovery phase, and the 16-week goat was clinically normal, with exercise tolerance similar to pretreatment at the conclusion of the study.

Serum Biochemistry

After 7 days of rayless goldenrod treatment (no recovery group), serum CK, AST, ALT, and LDH activities were all significantly increased from pretreatment and control activities (table 1). During recovery, individual animals often had elevated enzyme activities, especially the CK activities, but the mean of activities was not significantly different than control goats. However, individual animal results, especially the CK activities, tended to remain elevated as the recovery goats improved and were once again able to have near normal treadmill performances and recoveries. By 8 and 16 weeks post treatment both remaining animals had enzyme activities similar to pretreatment.

Necropsy and Histologic Studies

Necropsy findings of the goats necropsied immediately after dosing included swelling and pallor of nearly all skeletal muscles. The large appendicular muscles (semimembranosus, semitendinosus, biceps femoris, gluteus medius, quadriceps femoris, and triceps brachii) were most often severely affected. The hearts had minimal pale streaking in the myocardium. The livers of these animals were swollen and red. No significant gross lesions were found in the control goats that were dosed with ground hay. The goats necropsied 1, 2, and 4 weeks after treatment had minimal skeletal muscle swelling and pallor. The other tissues including the hearts appeared normal. Animals necropsied on weeks 8 and 16 were grossly normal.

Histologic studies of goats that were euthanized and necropsied immediately after dosing demonstrated extensive monophasic degeneration and necrosis of skeletal muscle (figure 2). The lesions were most severe and widely distributed in the large appendicular muscles. Affected skeletal muscles were degenerative and necrotic characterized by myofiber swelling, loss of striation, hypereosinophilia, clumping and disruption of sarcoplasmic contents, monocytic inflammation with

Table 1. Selected mean serum biochemical data from recovering goats dosed with rayless goldenrod to obtain benzofuran ketone dosages of 40 mg/kg BW/day for 7 days[*]

	Pretreatment[II]	No Recovery	1 Week	2 Weeks	3 Weeks	4 Weeks
Creatinine Kinase (CK < 350 U/L[I])	215±90[a]	11,952±8497[b]	748±447[a]	481±296[a]	330±99[a]	777±38[a]
Aspartate aminotransferase (AST < 125 U/L[I])	130±51[a]	4306±1869[b]	1418±421[a]	581±509[a]	338±267[a]	351±36[a]
Alanine amminotransferase (ALT < 55 U/L[I])	43±7[a]	452±205[b]	470±100[a]	193±62[a]	107±35[a]	87±19[a]
Lactate dehydrogenase (LDH < 1560 U/L[I])	1112±307[a]	9665±2961[b]	3650±721[c]	1960±1057[ac]	1438±1052[a]	1920±1021[ac]

* Data are reported as means ± standard deviation. Different means ($P < 0.05$) between groups are indicated with superscript letters. Results from animals after 8 and 16 weeks of recovery are not included as there were insufficient numbers for statistical comparison and the activities were similar to pre-treatment measurements.

[I] Estimates of normal range were determined as 2 SD from mean values of controls and pretreatment samples. These ranges are laboratory and assay specific.

[II] Mean and standard deviation of animals on initial treatment day.

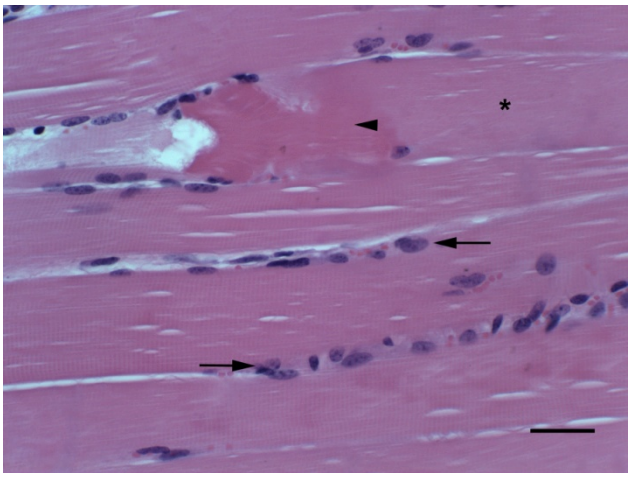

Figure 2. Photomicrograph of the quadriceps femoris muscle of a goat treated with rayless goldenrod (*Isocoma pluriflora*) to obtain a benzofuran ketone dosage of 40 mg/kg BW/day for 7 days. Note the extensive edema (*), myonecrosis with clumping of sarcoplasmic proteins (arrowhead), and minimal monocytic inflammation with nuclear proliferation (arrow). H&E, bar = 60 µm.

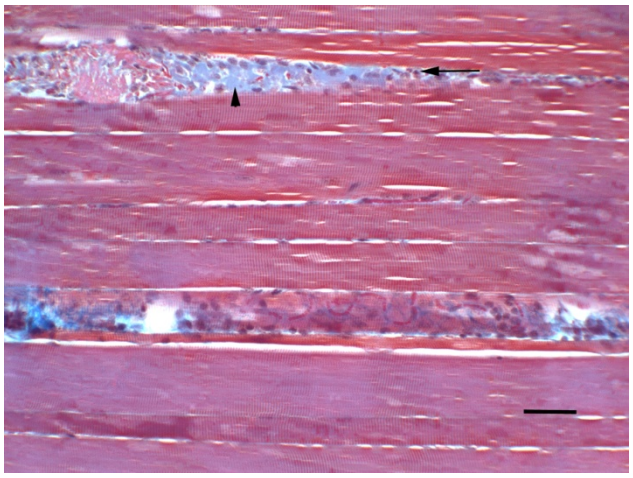

Figure 3. Photomicrograph of the quadriceps femoris muscle of a goat treated with rayless goldenrod (*Isocoma pluriflora*) to obtain a benzofuran ketone dosage of 40 mg/kg BW/day for 7 days. This section is stained with Mason's trichrome to demonstrate the minimal interstitial collagenous structure. Most of the positively staining material is fibrin (arrowhead) with adjacent monocytic inflammation (arrow). H&E, bar = 30 µm.

debris filled macrophages and proliferation of myocyte nuclei (figure 2). In these foci of myocyte necrosis there was minimal proliferation of fibroblasts with small amounts of collagen deposition (figure 3). The myocardial lesions were less severe and composed of mild myofibrillar swelling with loss of striations (figure 4). No lesions were identified in any of the other non-muscular tissues that were examined or in any of the tissues of the control animals.

The skeletal muscle lesions in the goats that were allowed to recover for 10 days and 2 weeks had degenerative and necrotic changes very similar to those seen in the goats that were euthanized directly after treatment. These lesions were characterized by myocytes in various stages of degeneration and necrosis with marked clumping of sarcoplasmic proteins and collapse of myofiber structure (degeneration phase, figure 5A).

In animals that were allowed 4 weeks to recover, the acute changes of swollen myocytes with loss of striation and hypereosinophilia were less common. Additionally, there was significantly more inflammation that was primarily composed of debris-filled macrophages and lymphocytes (inflammatory phase, figure 5B). The myocytes were also mildly regenerative as seen by proliferation of myocyte nuclei. There was also mild fibrosis with proliferation of fibroblasts and interstitial edema.

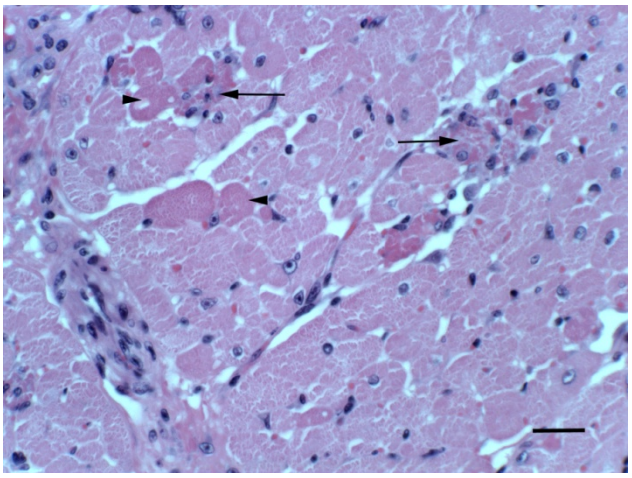

Figure 4. Photomicrograph of the papillary muscle from the left ventricle of a goat treated with rayless goldenrod (*Isocoma pluriflora*) to obtain a benzofuran ketone dosage of 40 mg/kg BW/day for 7 days. Notice the swelling and hypereosinophilia of many myocytes (arrow). H&E, bar = 30 µm.

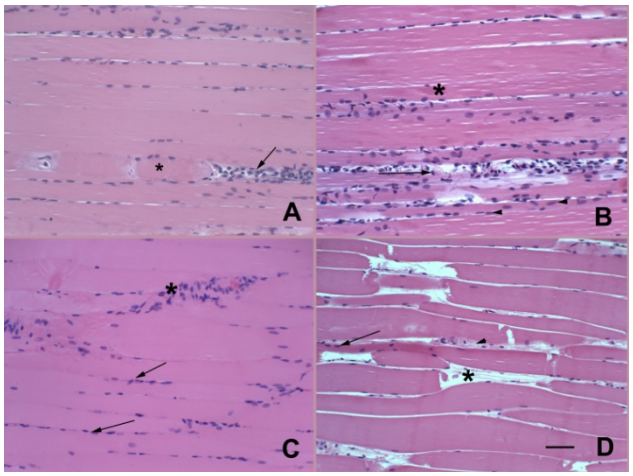

Figure 5. Photomicrographs of the quadriceps femoris muscle of a goat treated with rayless goldenrod (*Isocoma pluriflora*) to obtain a benzofuran ketone dosage of 40 mg/kg BW/day for 7 days. Plate A: This photomicrograph is from a goat that was allowed to recover for 2 weeks. Note the continuing myocyte degeneration and necrosis with sarcoplasmic hypereosinophilia, and coagulation of sarcoplasmic proteins (*). Also, notice the expansion of inflammatory cells that are mostly debris-filled macrophages (arrow). Plate B: This photomicrograph is from a goat that was allowed to recover for 4 weeks. Notice the occasional focus of myocyte necrosis with its associated coagulation of sarcoplasmic proteins (*). The inflammatory cells at this time are more numerous and are composed of debris-filled macrophages with fewer numbers of lymphocytes and plasma cells (arrow). There are increased numbers of proliferative or hyperplastic myocyte nuclei in these sections also. Plate C: This photomicrograph is from a goat that was allowed to recover for 8 weeks. In this section foci of myocyte necrosis are rare. The inflammatory cells at this time are more numerous and are composed of debris-filled macrophages with fewer numbers of lymphocytes and plasma cells (*). There are numerous proliferative or hyperlastic myocyte nuclei that often form prominent rows in bands of myocytes. Plate D: This photomicrograph is from a goat that was allowed to recover for 16 weeks. Notice there is no myocyte necrosis, and small clusters of inflammatory cells are rare (not pictured). The myocytes are variably sized, and most have prominent cross-striation. Some areas have prominent interstitial edema (*). All sections were stained with H&E and taken at the same magnification—bar = 30 µm.

The hearts and other tissues from these animals were histologically normal.

The lesions seen at 8 weeks were similar as the predominant change was myocyte regeneration (regenerative phase, figure 5C). The regeneration was characterized by increased numbers of enlarged hyperplastic myocyte nuclei and prominent nuclear rowing with collapse and macrophage removal of sarcoplasmic proteins. There was little evidence of continued myocyte degeneration. There was also less inflammation and the inflammatory component remaining was primarily composed of macrophages that often contained small fragments of cellular debris. Throughout these muscles were also small foci of fibroblast proliferation and extracellular collagen deposition.

After 16 weeks, there was no evidence of myocyte degeneration or necrosis and the inflammation was minimal and patchy. Many appendicular muscles of this animal had increased variation in myocyte size and some had some mild interstitial edema (remodeling phase, figure 5D). There were no other significant histologic lesions in tissues other than skeletal muscle in the goats that were necropsied 4, 8, and 16 weeks after treatment.

Discussion

Rayless goldenrod is a known toxic plant and has sporadically poisoned livestock for over 100 years (Marsh 1926, Burrows and Tyrl 2001). Though various toxins such as the benzofuran ketones—tremetone, dehydrotremetone, and 3-oxyangeloyltremetone—have been implicated in rayless goldenrod poisoning, definitive identification of the cause of myodegeneration or the mechanism has not been proven (Couch 1927, 1930; Zalkow et al. 1962; Beier et al. 1993; Lee et al. 2009). The variety of clinical and histologic disease in different species—and with different plant collections and phenotypes—suggests that poisoning may produce a spectrum of clinical diseases. Additional work is

needed to determine which compounds are toxic, which toxins produce what lesions, which plant varieties and phenotypes contain toxins, and the conditions of poisoning for different livestock species.

As seen in previous studies, we found that the rayless goldenrod we used in these studies consistently poisoned Spanish goats. Using doses of ground plant material to obtain dosages of 40 mg/kg benzofuran ketones/day for 7 days, all study animals were poisoned. The affected animals developed clinical signs and histologic lesions that were mirrored by increased serum enzyme activities of CK, LDH, AST, and ALT (table 1) similar to those described in previous studies (Stegelmeier et al. 2010). Although all of these activities at 7 days after dosing were different from baseline and control animals, differences disappeared as animals recovered, perhaps because of variability and the small sample size. Many of the recovery animals had high serum enzyme activities, especially CK activities, but high variability. The serum biochemical results seemed to show a relationship with the histologic myocyte degeneration and necrosis similar to previous studies (Stegelmeier et al. 2010).

The initial histologic skeletal myonecrosis was monophasic and acute with minimal nuclear proliferation and fibrosis (necrosis phase, figure 2). The minimal fibroblast proliferation and collagen accumulation are likely indicative of early fibrosis (figure 3). However, the lesions in early recovery animals were much more variable with more severe inflammation and fibrosis. These primarily inflammatory and fibrotic changes were most pronounced in the animals that were allowed to recover for 2 weeks. These lesions were much more difficult to classify because they contained patchy areas of continued degeneration and necrosis and other areas with more inflammation and early regeneration. Consequently, the degeneration and necrosis in the 10-day and 2-week animals would most likely be classified as polyphasic and regenerative. The later recovery animals were regenerative with some fibrosis. The 16-week animal was unique as most of the inflammation and cellular response had resolved, but there were foci or residual fibrosis and there was marked variation of myofiber size. Additional work including morphometry studies is needed to confirm these changes.

The myocardial lesions were relatively subtle and quickly resolved (figure 4). Previous studies report that doses above 1.0% of BW plant material were required to develop cardiac lesions (Stegelmeier et al. 2010). In this study, poisoned animals develop swelling and hypereosinophilia of cardiac myocytes. This appears to be a reversible degenerative change as it quickly resolved in the recovery animals with minimal secondary changes. As previously suggested, the doses we used were probably not high enough or of long enough duration to produce permanent myocardial damage. It has been suggested that all horses and young animals are more susceptible to the cardiotoxic effects of white snakeroot and rayless goldenrod (Kingsbury 1964, Burrows and Tyrl 2001). More information is needed to determine if there are age and species differences especially relating to cardiotoxicity. This may be especially important; under some conditions, these lipophilic toxins are preferentially excreted in milk. Subsequent transmammary poisoning of highly susceptible nursing animals could result in permanent cardiac damage that would impair subsequent production and performance.

In conclusion, we have shown that oral gavage of rayless goldenrod in goats to obtain dosages of 40 mg benzofuran ketone /kg bw/day for 7 days induced skeletal and cardiac muscle degeneration and necrosis. The cardiac lesions were mild and quickly resolved within 10 days of poisoning. The skeletal muscle lesions were more severe and resolved through stages of inflammation (10 days and 2 weeks), regeneration, and fibrosis (4 and 8 weeks). The resolution phase (16 weeks) was characterized by minimal residual lesions of focal residual inflammation with small foci of fibrosis and variation in myofiber size. These findings indicate that poisoning causes significant clinical and histologic skeletal muscle changes that all but resolve within 3 months of poisoning. The significance or persistence of the residual fibrosis and myofiber change is unknown. Certainly, such changes are likely to alter function of performance and working animals. Many questions of rayless goldenrod poisoning remain unanswered and additional studies are needed to determine the relative toxicity of individual benzofuran ketones to better define the specific pathogenic mechanism of their toxicity, and to determine the impact and long-term sequelae of poisoning in other livestock species.

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Cerebellar Cortical Degeneration in Cattle Poisoned With *Solanum* spp. in South America: An Epidemiological, Clinicopathological, Pathological, and Toxicological Review

José M. Verdes[1 *], Franklin Riet-Correa[2], Rosane M.T. Medeiros[2], Antonio Moraña[1], Daniel Battes[1], Virginia Dehl[1], Claudio Borteiro[1], Eduardo J. Gimeno[3], Fabiano J.F. Sant'Ana[4], Claudio S.L. Barros[5], and Severo S. Barros[5]

[1]University of the Republic, Molecular and Cellular Biology (Biophysics) and Pathology Departments, Faculty of Veterinary, Montevideo, Uruguay
[2]Federal University of Campina Grande, Center for Health and Rural Technology, Patos, PB, Brazil
[3]National University of La Plata, Faculty of Veterinary Science, La Plata, Argentina
[4]Federal University of Goiás, Laboratory of Veterinary Pathology, Jataí, GO, Brazil
[5]Federal University of Santa Maria, Laboratory of Veterinary Pathology, Santa Maria, RS, Brazil

[*]Corresponding author: J.M. Verdes, jmverdes@fvet.edu.uy

Abstract

Cattle that consume *Solanum bonariense* L (= *Solanum fastigiatum* Willd.) or *Solanum paniculatum* L. develop a typical cerebellar cortical degeneration characterized by periodic episodes of ataxia, hypermetria, hyperesthesia, head and thoracic limb extension, opisthotonus, nystagmus, and falling to the side or backward. Histological lesions include vacuolation, degeneration, and loss of Purkinje cells. Axonal spheroids, microcavitations, and other changes of Wallerian degeneration in cerebellar granular layer and white matter are also observed. Neurotoxic compounds in *Solanum* spp. causing neurologic dysfunction in ruminants were not definitively elucidated. The same Solanaceae species are extensively used with culinary purposes or for the treatment of liver and gastrointestinal disorders as hangovers in humans. In the present paper, we review the epidemiology, clinical signs, and pathological hallmarks of poisoning by *Solanum* —*S. bonariense* L. (=*S. fastigiatum* Willd.) and *S. paniculatum*—with emphasis in histopathology, ultrastructural, and lectin- and immuno-histochemical changes in spontaneous and experimentally poisoned cattle in South America. The current knowledge of the pathogenesis of these bovine cerebellar cortical degenerations is discussed, and some advances in botanical and toxicological aspects of these Solanaceae species are presented, taking into account the potential risk of human poisoning.

Keywords: cerebellar degeneration, diseases of cattle, poisonous plants, Solanacae

Introduction

From the beginning of the16th century when cattle were first introduced into South America, the use of autochthonous plants as forage contributed to livestock losses from toxic plants that were novel to Spanish and Portuguese settlers. To overcome this drawback, the settlers had to recognize and consider these potential risks to minimize animal deaths, and in some cases, learn about the aboriginal practices or develop new strategies to avoid plant poisoning. A good example of these practices is the description of *Baccharis coridifolia* (mio-mio) poisoning and control by the Spanish Jesuit chronicler Bernabé Cobo (Cobo 1653).

At present, poisonous plants still constitute a very important problem causing direct and indirect losses to farmers in South America. An estimation of

total cattle losses obtained from different regional veterinary diagnostic laboratories from Brazil and Uruguay indicates an annual mortality rate of 5 percent, of which 10 to 14 percent is due to poisonous plants (Riet-Correa and Medeiros 2001). A recent survey of cattle necropsies examined at the Laboratory of Veterinary Pathology of Federal University of Santa Maria from 1990 to 2005 (2,912 cases) indicates that in 15.83 percent of the cases, the cause of death was attributed to the ingestion of poisonous plants (Rissi et al. 2007). *Solanum fastigiatum* Willd. *var. fastigiatum* ("Jurubeba") causes serious intoxication problems in cattle in Rio Grande do Sul, southern Brazil (Rissi et al. 2007, Sant'Ana et al. 2011), causing an irreversible cerebellar disorder (Riet-Correa et al. 1983, Zambrano et al. 1985, Barros et al. 1987, Paulovich et al. 2002, Rech et al. 2006, Sant'Ana et al. 2011). Similar cerebellar syndromes were described for *Solanum bonariense* L. ("Naranjillo") in Uruguay and Argentina (Podestá et al. 1971, Riet-Correa et al. 1983, Verdes 2006, Verdes et al. 2006, Verdes et al. 2007, Verdes et al. 2010, Odriozola et al. 2012) and for *Solanum paniculatum* L. ("Jurubeba") in Brazil (Barros et al. 1987; Medeiros et al. 2004; Guaraná et al. 2011a,b). These Solanaceae species belong to a genus distributed worldwide that encompasses other well-recognized toxic species that cause multiple disease conditions and death by different mechanisms, and that are especially abundant in tropical and subtropical regions of Central and South America (Baker et al. 1989). The intoxication has been experimentally reproduced in cattle (Riet-Correa et al. 1983, Barros et al. 1987, Medeiros et al. 2004, Verdes et al. 2006).

As in other cases of *Solanum* intoxication that cause cerebellar degeneration in South Africa, the United States, and Australia (Piennar et al. 1976, Menzies et al. 1979, Bourke 1997, Porter et al. 2003), the main pathophysiological features of South American species are the vacuolation of the perikaria in Purkinje cells followed by loss of these neurons and the presence of axonal spheroids, as well as microcavitations in the cerebellar granular layer and/or white matter (Riet-Correa et al. 1983; Barros et al. 1987; Medeiros et al. 2004; Verdes et al. 2006; Guaraná et al. 2011a,b).

On the other hand, *S. paniculatum* and *S. fastigiatum* are extensively used for culinary purposes, in alcoholic beverages (figure 1), or as infusions in Brazilian folk medicine for the treatment of liver and gastrointestinal disorders from excess alcohol consumption in humans (Vieira et al. 2010). They are potent inhibitors of gastric acid secretion (Mesia-Vela et al. 2002), and their aqueous extract has antioxidant and hepatoprotective activity in mice (Sabir and Rocha 2008).

Figure 1. *Solanum* sp. ("Jurubeba") is used often for culinary purposes or in alcoholic beverages. Left, "Jurubeba" fruit sauce offered as a condiment in a traditional self-service restaurant in Goiás near Brasilia, Brazil. Right, a bottle of "Jurubeba" wine purchased in the interior of the State of Pernambuco, Brazil.

Until recently the neurotoxic compounds in *Solanum* spp. causing neurologic dysfunction in ruminants were not definitively elucidated (Baker et al. 1989, Verdes et al. 2007, Guaraná et al. 2011b). However, different substances or fractions were characterized from some of these South American species, stimulating bioassay-guided studies in animal models (Mesia-Vela et al. 2002, Ruiz-Diaz et al. 2004, Higa et al. 2006, Sabir and Rocha 2008).

Although rodents seem to be adequate biomodels to test some pharmacological effects of *Solanum* spp., at the present time, no susceptible laboratory animals have been found to reproduce cerebellar dysfunction similar to that reported in cattle (Riet-Correa et al. 1983, Zambrano et al. 1985, Ruiz-Diaz et al. 2004). Nevertheless, sheep (Zambrano et al. 1985) and goats (Verdes et al. unpublished results) seem to be alternative biomodels to study this neurodegeneration.

The identification and characterization of neuroactive principles present in aerial parts of *Solanum* spp., as well as its ruminal by-products, could become a key step to definitively clarify its specific toxicological pathways towards its target, the cerebellar Purkinje cells. This would contribute to an understanding of the pathogenesis of these acquired cerebellar degenerations in ruminants and also to the safe use of these plants as natural or folk medicine for humans.

The objective of the present paper is to review the epidemiology, clinical signs, and pathological hallmarks of poisoning by *Solanum–S. bonariense* L. (=*S. fastigiatum* Willd.) and *S. paniculatum*–with emphasis in histopathology, ultrastructural, lectin- and immuno-histochemical changes in spontaneous and experimental poisoned cattle in South America. The current knowledge of the pathogenesis of these bovine cerebellar cortical degenerations is discussed, and some advances in botanical and toxicological aspects of these Solanaceae species are presented.

Epidemiology and Clinicopathology of the Poisoning in Cattle and Animal Models

The spontaneous disease caused by *Solanum* spp. affects cattle older than 8 months of age of various breeds, but dairy cattle and crossbred animals are most affected. Morbidity varies from 1 to 25 percent. Deaths are uncommon and are associated with progressive weakening, drowning accidents, or traumatic injuries; in some specific cases, mortality can reach 20 percent (Guaraná et al. 2011b). Farmers usually sell affected animals for slaughter when clinical signs are observed. No seasonal differences were detected in the incidence of the disease (Riet-Correa et al. 1983).

A major clinical feature of affected cattle is the occurrence of periodic attacks in which CNS dysfunction leads to falls and an inability to rise, without loss of consciousness, lasting up to 1 minute; most animals appear normal between episodes. Other clinical signs include ataxia, hypermetria, hyperesthesia, staggering gait, muscle tremors, head and thoracic limb extension, opisthotonus, nystagmus, and in those animals most severely affected, falling to the side or backward. Nervous signs occur spontaneously or are induced when affected animals became excited or intentionally stressed, for instance with the head-raising test (Pienaar et al. 1976, Riet-Correa et al. 1983, Verdes et al. 2006, Guaraná el al. 2011a). A few animals show permanent neurological signs, including dysmetria and a "star gazing" attitude. In affected animals, serum levels of AP, AST, and GGT are usually normal (Riet-Correa et al. 1983, Verdes et al. 2006).

The poisoning has been experimentally reproduced in cattle, inducing clinical signs similar to those observed in the spontaneous intoxication (Riet-Correa et al. 1983, Zambrano et al. 1985, Barros et al. 1987, Medeiros et al. 2004, Verdes et al. 2006, Guaraná el al. 2011a) (table 1). All experimental studies clearly demonstrate that cattle had to consume considerable quantities of the plant, over 76 to 260 days, in order to show clinical signs (table 1). Thus, the occurrence of clinical signs is more likely when pastures invaded by *Solanum* spp. are overgrazed (Riet-Correa et al. 1983, Zambrano et al. 1985, Barros et al. 1987, Medeiros et al. 2004, Guaraná el al. 2011a) or when the grass in the affected paddocks was previously mowed before introduction of the animals. This situation promotes the sprouting of *Solanum* spp. in the pastures, which likely facilitates their intake by cattle (Guaraná el al. 2011b).

In sheep, similar cerebellar lesions as those described for cattle were observed after administration of a commercial ration containing 20 percent of dry plant for 202 to 370 days (total dose 0.43 kg of dried leaves/kg BW). However, none of the guinea pigs, rabbits, or rats were intoxicated after being given an oral dose via commercial ration containing 10 percent of dry plant for 120 days (Zambrano et al. 1985). Recently, similar cerebellar lesions to those in cattle and sheep were observed in goats after oral administration of dry leaves (Verdes et al. unpublished results).

Finally, it is important to note the existence of several diseases of cattle characterized by signs of cerebellar insufficiency, some of which also produce degeneration and vacuolation of Purkinje cells and axonal spheroids as observed in *Solanum* spp. poisoning (table 2). A very intriguing characteristic of cerebellar cortical degeneration induced by *Solanum* spp. is the highly selective damage done to Purkinje cells. The basis for this selectivity remains unknown; the identification of the stored material within these neurons or determination of the neuroactive substances present in these shrubs would help to clarify this aspect (Riet-Correa et al. 1983, Verdes 2006).

Pathology, Histopathology, Transmission Electronic Microscopy (TEM), and Lectin Histochemistry

Natural cases of *Solanum* poisoning usually do not present significant macroscopic lesions (Riet-Correa et al. 1983, Verdes et al. 2006). However, Rech et al. (2006) observed a case of encephalic traumatic subdural hemorrhage and another case with gross atrophy of the cerebellum out of 19 cases reported. Similarly, Guaraná et al. (2011b) found gross atrophy of the cerebellar grey matter in one of two cases of *S. paniculatum* poisoning, and Sant'Ana et

Table 1. Summary of different strategies used to induce experimental cerebellar cortical degeneration in cattle with *Solanum* spp.

Species	Dosage schedule and method used	Cumulative dose	Days before first clinical signs	Reference
S. fastigiatum Willd. (1809) var. *fastigiatum**	0.4 kg/day of dried leaves via rumen cannula	0.18 kg of dried leaves/kg BW	155 days	Riet-Correa et al. 1983
S. fastigiatum Willd. (1809) var. *fastigiatum**	Oral administration of commercial ration containing 10% of dry plant	0.14 kg of dried leaves/ kg BW	76 days	Riet-Correa et al. 1983
Solanum sp.**	Green leaves *ad libitum* for 639 days	0.94 kg of green leaves/ kg BW	260 days	Barros et al. 1987
Solanum sp.**	5 g/kg BW of dried leaves, via rumen cannula (5 days/week)	0.17 kg of dried leaves/kg BW	106 days	Barros et al. 1987
Solanum paniculatum L. (1762)	5 g/kg BW of dried leaves, via rumen cannula (5 days/week)	Not showed	93 to 102 days	Medeiros et al. 2004
Solanum bonariense L. (1753)*	Green leaves at 1% BW, via rumen cannula, daily	1.024 kg of green leaves/kg BW	128 to 136 days	Verdes et al 2006
Solanum paniculatum L. (1762)	Oral administration of commercial ration containing 3g of dry plant/kg BW during 3 months and after that, 4g of dry plant/kg BW during 1 month more	Not showed	120 days	Guaraná et al. 2011a

* *S. bonariense* L. (=*Solanum fastigiatum* Willd.) (Chiarini et al. 2007, Wagstaff 2008).
** Later identified as *S. paniculatum* L. (Franklin Riet-Correa, unpublished data).

Table 2. Diseases with clinical signs and pathology similar to *Solanum* spp. poisoning in cattle

In utero infection with BVD (at birth)
Cerebellar abiotrophia (hereditary, appears up to 8 months of age)
Cerebellar cortical atrophy (unknown cause, up to 8 months of age)
Familial convulsions in Angus cattle (inherited)
Mannosidosis or pseudolipidosis of Angus cattle (inherited)
GM1 gangliosidosis of Friesian cattle (inherited)
Others inherited Lysosomal Storage Diseases*
Lysosomal Storage Diseases acquired by plant poisonings (i. e.: *Swainsona* spp, *Astragalus* spp., *Oxytropis* spp., *Sida* spp., and *Ipomoea* spp.)*

*Cell vacuolation are present in visceral tissues and in other regions of CNS

al. (2011) reported a moderate atrophy of the cerebellum in 2 out of 33 spontaneously poisonedcattle. Other traumatic lesions like hip luxation are probably caused by traumatic injury during sporadic poisoning episodes (Riet-Correa et al. 1983).

The microscopic lesions are almost always localized only in the cerebellum. Vacuolation of the perikarya is present in Purkinje neurons (figure 2). Eosinophilic, round, homogeneous, occasionally fine granular axonal spheroids and microcavitations are found in the granular layer and cerebellar white matter (Riet-Correa et al. 1983, Verdes et al. 2006, Guaraná et al. 2011b) (figure 3). The quantification of Purkinje cells in spontaneous cases shows a decrease in number (Verdes et al. 2006, Rech et al. 2006) and in association with a consequent decrease in the thickness of the molecular layer (Rech et al. 2006).

In the experimental poisoning of cattle, the decrease in numbers of Purkinje cells is associated with an increment of axonal spheroids and microcavitations in the cerebellar granular layer and white matter (Verdes et al. 2006). The severity of these alterations are associated with the quantity of plant consumed (Zambrano et al. 1985, Verdes et al. 2006).

Other interesting features of this plant poisoning are the proliferation of Bergmann glia in the cerebellar cortex or gliosis in the cerebral cortex and pons (Riet-Correa et al. 1983, Verdes et al. 2006, Guaraná et al. 2011b). Axonal spheroids and some vacuolated neurons are observed in fastigial,

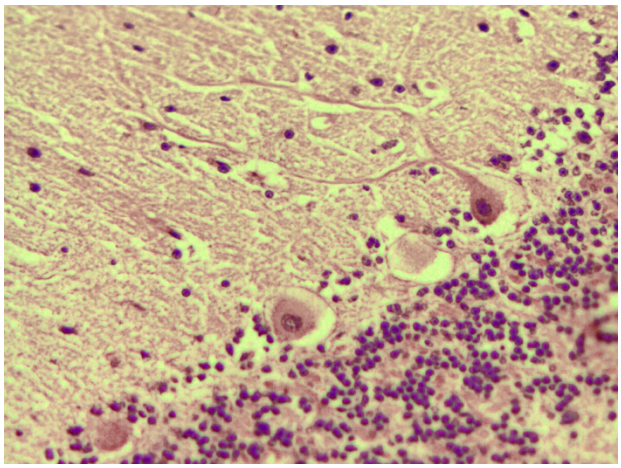

Figure 2. Cerebellar cortex of a steer experimentally poisoned with *Solanum bonariense* L. Note Purkinje neurons showing vesiculated perikaryon and nuclear displacement. HE stain.

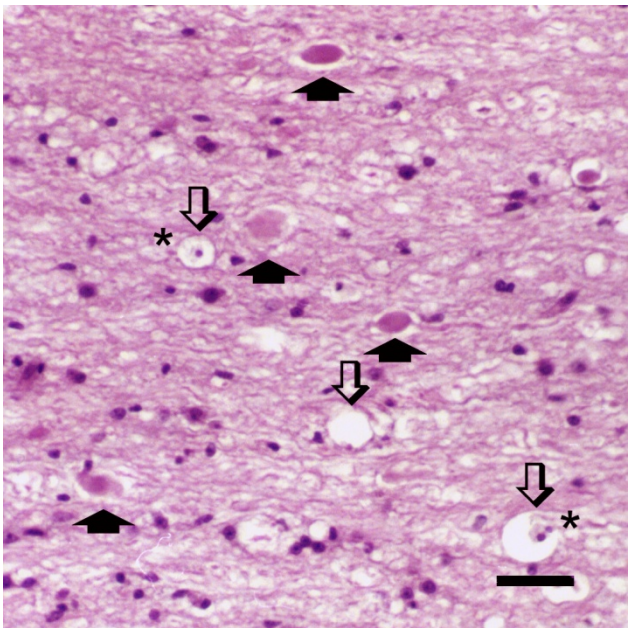

Figure 3. Cerebellar white matter of spontaneously poisoned heifer showing axonal spheroids (black arrows) and macrophages (asterisks) within some microcaviations (empty arrows). HE stian. Bar=70µm.

interposital, and lateral cerebellar nuclei, and more rarely found in the caudal colliculi (Riet-Correa et al. 1983), gracillis nucleus (Guaraná et al. 2011b), or in other brain stem nuclei (Odriozola et al. 2012). Degenerative changes in the cerebellar cortex suggest a progressive demyelination of white matter by Wallerian degeneration (Riet-Correa et al. 1983, Verdes et al. 2006), which was confirmed by Kluver-Barrera and Bielchowsky staining methods (Verdes et al. 2011).

Gangliosides and glycolipids are particularly abundant in neurons, requiring a continual

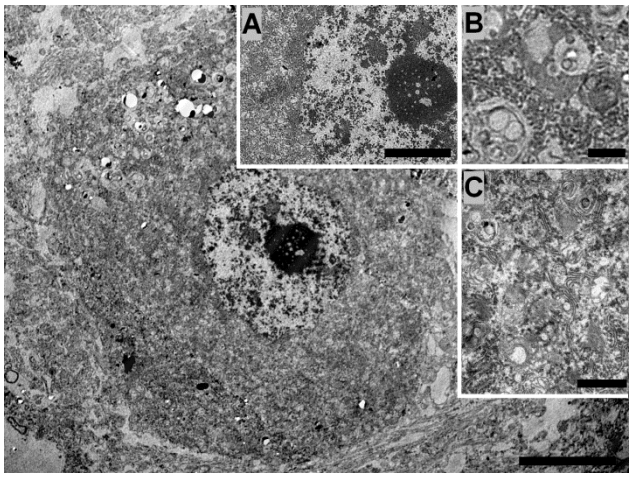

Figure 4. A TEM image of a Purkinje cell in a steer experimentally poisoned by *Solanum bonariense*. The selected neuron shows damage in organelles present in the perikaryon of the cell, but no signs of nuclear apoptosis can be seen. Bar = 10000 nm. A. Magnification of the nuclear area where disaggregation of granular and fibrillar elements can be observed in the nucleolus. Bar = 5000 nm; B. Magnification of the perikaryon of the same cell showing typical extensive vacuolation and vesicles filled with several membranous bodies. Bar = 1000 nm; C. Lamellar arrays of endoplasmic reticulum and myelin figures filled with membranous debris are observed. Bar = 500 nm.

degradation and resynthesis of new molecules. Typical gangliosidoses are inherited lysosomal storage diseases characterized by an altered expression of lysosomal enzymes responsible to hydrolyze gangliosides, resulting in storage of these incomplete degradative by-products in neurons and others cells. This causes a lysosomal overload of this undigested material and results in neurological dysfunction and other diseases (Riet-Correa et al. 1983, Smith 2006, Guaraná et al. 2011b). A quantification of gangliosides in the cerebellum, especially in Purkinje cells, of normal and poisoned cattle is necessary to confirm that cerebellar cortical degenerations are associated with the accumulation of these substances. GD1α, a sialoganglioside highly expressed in Purkinje cells, could be a potential candidate to confirm or refute this hypothesis (Furuya et al. 1996, Verdes 2006).

The axonal spheroids are the result of myelinated axon enlargement, with many heterogeneous membranous organelles filled with residual bodies and swollen mitochondria (Riet-Correa et al. 1983, Barros et al. 1987, Verdes et al. 2006). The progressive increase in the ratio of axoplasm to myelin confirms demyelination by Wallerian degeneration in affected axons (Barros et al. 1987, Verdes et al. 2006). The microtubules and

neurofilaments are markedly altered in affected Purkinje cells, some dendrites, and axonal spheroids (Barros et al. 1987, Verdes et al. unpublished results). Also, a great concentration of mitochondria is present in swollen axons, as well as in some swollen dendrites of Purkinje cells in the cerebellar molecular layer (Barros et al. 1987).

In cattle poisoned with *Solanum bonariense* (= *Solanum fastigiatum*), the lectin binding pattern of the stored material present in affected Purkinje cells demonstrates accumulation of β- (1-4)-D-N-acetyl-glucosamine, α-D-mannose, α- D-glucose, D-mannose, D-glucose, D-N-acetyl chitobiose, and N-acetyl lactosamine residues (Sant'Ana et al. 2011). A similar pattern is found in glycolipid storage diseases (Paulovich et al. 2002, Sant'Ana et al. 2011) or in those conditions detected in plant-induced α-mannosidosis, including poisonings by plants of the genera *Swainsona, Oxytropis, Astragalus, Sida*, and *Ipomoea.* However, in the latter cases, there is additional vacuolization in pancreatic, liver, and kidney epithelial cells (Alroy et al. 1984, 1985; Driemeier et al. 2000; Armién et al. 2007; Cholich et al. 2009; Sant'Ana et al. 2011).

Techniques to Identify the Pathogenetic Basis of These Cerebellar Cortical Degenerations

The decreased cytoskeletal components, particularly neurofilaments and neurotubules, observed by Barros et al. (1987) and the ribosomal disaggregation observed by Verdes et al. (2006) suggest that alteration of protein synthesis may occur in affected neurons. Protein synthesis alteration, as well as the concomitant cytoskeleton derangement, could play a role in the pathogenesis of these neurodegenerations with subsequent altered cell-specific axonal transport determining neuronal death (Verdes 2006, Verdes et al. 2006). The identification of the basic nature of these cytoskeletal alterations is an important step towards the understanding of underlying disease processes. To identify and characterize cytoskeletal alterations in the perikaryon of Purkinje cells in intoxicated cattle, immunohistochemistry against different cytoskeletal proteins is currently being used. Immunoreactivity for phosphorylated neurofilament protein β-tubulin and affinity reaction against phalloidin revealed an altered cellular distribution of these interconnected components of the neuronal cytoskeleton in poisoned cattle (Verdes et al. unpublished results). These preliminary results seem to indicate that the altered cytoskeleton is somehow related to the miss-accumulation of membrane-bound cytoplasmic vesicles seen in affected neurons. Further investigation is needed to understand whether cytoskeletal alterations occur in the pathogenic cascade as a primary step leading to the vesicular miss-accumulations, or if they are a secondary/downstream event (Verdes et al. 2006).

Another feature that suggests the occurrence of metabolic stress in degenerating Purkinje cells is the altered immunohistochemical pattern of conjugated ubiquitin that indicates the inhibition of the ATP-dependent hydrolytic ubiquin-proteasome system, a non-lysosomal hydrolytic pathway responsible for digesting misfolded proteins that is altered in human neurodegeneration (Glickman and Ciechanover 2002, Lindsten et al. 2002, Klimaschewski 2003, Korhonen and Lindholm 2004, Verdes 2006). Different reports suggest that CbD28k is a specific marker of Purkinje cells in both normal and degenerative cerebellums (Ishikawa et al. 1995, Haworth et al. 2006, Verdes et al. 2010). Moreover, in the cerebellum where rapid-firing neurons experience a high level of calcium influx, intracellular calcium is mainly regulated by calcium binding proteins (CaBPs). For example, some excitotoxic degenerative Purkinje cells may have a disruption of calcium-buffering systems.

Calbindin D 28k (CbD28k) is a CaBP highly expressed in Purkinje cells (Ishikawa et al. 1995, Haworth et al. 2006, Verdes et al. 2010). Its expression in a variety of acute and chronic disorders seems to have a neuroprotective role against degeneration (Krebs 1998, Bastianelli 2002, Clowry and McHanwell 2004), specifically against calcium-mediated excitatory amino acid neurotoxicity by reducing levels of intracellular free calcium (Ishikawa et al. 1995). This does not seem to be the case in *Solanum* spp. poisoning because CbD28K preserves its immunoreactivity in cell bodies, axons, and dendrites. The immunoreactivity of CbD28K is similar in both experimentally and naturally poisoned animals as well as in controls. Degenerative axons or spheroids in the cerebellar white matter are also CbD28k positive; neurons of the deep cerebellar nuclei are CbD28k negative; and the surrounding synaptic terminals are positive (Verdes et al. 2010).

Finally, using TUNEL apoptotic reagents to define if programmed cell death pathways are activated in Purkinje cells of *Solanum* spp. poisoned cattle, Verdes et al. (2010) demonstrated that apoptosis is not activated. Van der Lugt et al. (2010) reached similar conclusions with bovines intoxicated by *S. kwebense*. In the *S. bonariense* cases, the

absence of typical apoptotic signs in the nuclei of affected Purkinje cells using TEM (figure 3) also ruled out the occurrence of apoptosis. This would suggest that the changes observed in degenerative neurons were probably more indicative of necrosis, although this hypothesis must be confirmed by more specific methods (Verdes et al. 2010).

Botanical and Toxicological Aspects

S. bonariense L. (=*Solanum fastigiatum* Willd.)

Solanum bonariense L., as described by Linnaeus in 1753, was probably based on a specimen from Buenos Aires, Argentina; and *S. fastigiatum* was proposed by Willdenow in 1809, based on a specimen grown in Berlin, Germany, of unknown origin. Dunal (1852) defined a variety of typical *Solanum fastigiatum* Willd. (var. *fastigiatum*), describing *Solanum fastigiatum* var. *acicularium.* The differences between typical *S. fastigiatum* Willd., and its variety *acicularium* are not clear except that the variety is said to be much spinier, but this may not be a character of much taxonomic significance in these plants (Morton 1976, Chiarini et al. 2007). Both species are morphologically very similar (Morton 1976, Lombardo 1983, Riet-Correa et al. 1983), and recently Chiarini et al. (2007) and Wagstaff (2008) suggested that *S. fastigiatum* is a synonym of *S. bonariense* (figure 5).

S. bonariense is widely distributed in southern Brazil, northeastern Argentina, and Uruguay (Morton 1976, Lombardo 1983, Simões et al. 1998, Chiarini et al. 2007, Sabir and Rocha 2008). It also occurs as a naturalized weed in the Mediterranean region of Europe after its introduction as an ornamental plant (Pascual-Villalobos 1999, Bruneton 2001, Rita 2002). Plant extracts from European specimens have demonstrated insecticide activity (Pascual-Villalobos 1999).

Solanum bonariense leaves and roots are used in Brazilian medicine as a tonic or for the treatment of fever, anemia, erysipelas, hepatitis and other liver disease conditions, spleen disorders, uterine tumors, irritable bowel syndrome, chronic gastritis, and other digestive problems such as sluggish digestion, bloating, and flatulence (Riet-Correa et al. 1983, Sabir and Rocha 2008). The "jurubeba" leaf tea is a very common household remedy throughout Brazil for hangovers. The aqueous extract of the plant has antioxidant and hepatoprotective activity in mice with liver damage (Sabir and Rocha 2008). Phytochemical analysis has shown the presence of rutin, flavonoids, and glycosides in leaves (Higa et al. 2006). Specimens collected in farms affected by spontaneous cerebellar disease of cattle in Uruguay did not contain swainsonine or calystegines (R. Molyneux 2005, personal communication), but some specific alkaloids were obtained from alcoholic fractions of leaves (Ruiz-Diaz et al. 2004, M. Haraguchi 2012, personal communication). A complete botanical description of *S. bonariense* and some interesting details about its synonymy are given by Chiarini et al. (2007).

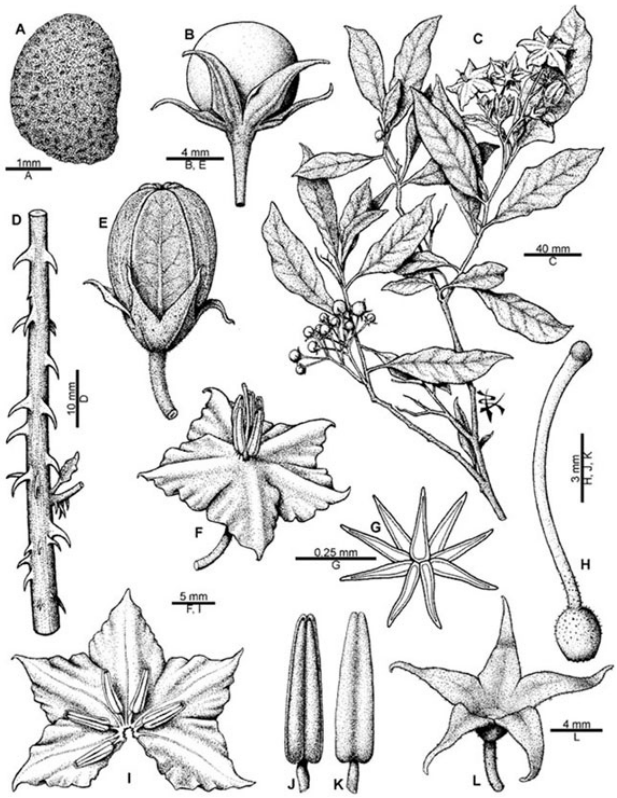

Figure 5. *Solanum bonariense* (D: Chiarini & Marini 642; A-C, E-L: Barboza et al. 1568). A: seed; B: fruit; C: branch with flowers and fruits; D: part of stem showing prickles; E: flower bud; F: flower; G: trichome sessile on the leaf lower surface; H: gynoecium; I: open corolla; J, K: anthers, ventral and dorsal views respectively; L: calyx. Courtesy of Chiarini et al. 2007, with permission of Gayana Botánica.

Solanum paniculatum L. (1762)

Solanum paniculatum L. was described by Linnaeus (1762) as a neotropical weed of very common occurrence in Brazil, Paraguay, Bolivia, and Argentina. It is used in folk medicine and for culinary purposes. Many species of the genus *Solanum* are known by local people as "jurubeba," but the species *S. paniculatum* is considered the authentic "jurubeba" (Corrêa 1984). The infusion prepared with "jurubeba" is a very common household remedy used throughout Brazil for

hangovers, because it exhibits antisecretory gastric properties (Vieira et al. 2010). Extracts of all parts of this plant are used as anti-inflammatory, antioxidant, molluscicidal, diuretic, and hepatoprotective agents. Many steroidal compounds have been isolated from this species; these alkaloids include jurubebine, jubebine, and solanine. The phytochemical analysis of *S. paniculatum* extracts showed variation according to the plant parts. Fructose, glucose, and galactose were detected in the fruits, and solanine was isolated from its roots and stems. Saponins were also identified in the roots of this species, including isojuripidine, isojurubidine, isopaniculidine, and jurubidine. Jurubidine, a sugar-free steroid obtained via acid hydrolysis of the glucoside jurubine, was also isolated from *S. paniculatum* roots. The alkaloids jurubebine and jubebine were identified in leaves and fruits (Vieira et al 2010).

The mouse is currently used as an animal model to study different pharmacological effects and to characterize folk medicine uses (Mesia-Vela et al. 2002, Vieira et al. 2010). No toxic signs were observed in mice following administration of different aqueous extracts up to 2 g/kg BW intraduodenally, which promoted antiulcer activity (Mesia-Vela et al. 2002).

Discussion

Different hypotheses about the pathogenic mechanism of this intoxication have been proposed for *Solanum* species. Riet-Correa et al. (1983) suggested that these plants cause an induced lysosomal storage disease, probably an acquired gangliosidosis or a neurolipidosis. In the case of normal Purkinje cells, the ganglioside GD1α appears to be highly expressed (Furuya et al. 1996). Its quantification in normal and poisoned cattle could be an interesting tool to clarify if these cerebellar cortical degenerations are acquired gangliosidoses (Verdes 2006).

The main feature of the intoxication is the selective damage to Purkinje cells. Barros et al. (1987) suggested that the disease is not a typical storage one, instead proposing a physico-chemical interaction between the active principle of the plant and normal lipids within neurons with concomitant formation of a complex that would be less susceptible to enzymatic degradation than normal lipidic compounds. Lectin histochemical patterns seem to support this hypothesis (Paulovich et al. 2002, Sant'Ana et al. 2011).

Ultrastructural studies in Purkinje cell perikarya also show alteration of the endoplasmic reticulum, a decrease in the number of neurofilaments and microtubules (Barros et al. 1987), ribosomal disaggregation, accumulation of electron-dense vesicles, and swollen mitochondria (Verdes et al. 2006). Similar alterations of the cytoskeleton and accumulation of vesicles are observed in axonal spheroids of granular layer and subcortical white matter (Barros et al. 1987, Verdes et al. 2006) and confirmed by immunohistochemistry (Verdes et al. unpublished results). These findings are in agreement with the hypothesis that alteration of protein synthesis and subsequent axonal transport disruption could play a role in the development of axonal spheroids and the pathogenesis of these cerebellar cortical degenerations (Verdes et al. 2006). Another fact that confirms the neuronal metabolic stress in these neurodegenerations is the presence of altered immunostaining against ubiquitin, supporting the hypothesis of inhibition of the ATP-dependent hydrolytic ubiquitin proteasome system (Verdes 2006). Degenerating Purkinje cells preserve calcium homeostasis and progressively die by necrosis (Verdes et al. 2010).

Available botanical, phytochemical, and clinicopathological evidence of this poisonous plant genera supports the view that both *S. bonariense* and *S. fastigiatum* are synonyms (Chiarini et al. 2007, Wagstaff 2008) or at least closely related species. Future work should address the use of sheep and goats as practical experimental models to study cerebellar cortical degeneration in ruminants, given the susceptibility of these domestic species and their smaller size.

The risk of human poisoning from *Solanum* spp. should also be considered and better characterized. The toxic compounds of *Solanum* spp. are still largely unknown, yet these plants are widely used as traditional medicines. The use of *Solanum* spp. in folk medicine is not recommended (Riet-Correa et al. 1983, Guaraná et al. 2011b).

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Weather and Plant Age Affect the Levels of Steroidal Saponin and *Pithomyces chartarum* Spores in *Brachiaria* Grass

Flavia G. Lima[1*], Mitsue Haraguchi[2], James A. Pfister[3], Victor Y. Guimaraes[1], Diogo D. F. Andrade[1], Carolina S. Ribeiro[1], Gustavo L. Costa[1], Ana Luiza L. Araujo[2], and Maria Clorinda S. Fioravanti[1]

[1]Federal University of Goias, Goiania, GO, Brazil
[2]Biological Institute, Sao Paulo, SP, Brazil
[3]USDA-ARS, Poisonous Plant Research Laboratory, Logan, UT, USA

*Corresponding author: Flavia G. Lima, flaviamedvet@yahoo.com.br

Abstract

Brachiaria species are cultivated worldwide in tropical and subtropical climates as the main forage source for ruminants. Numerous tropical and warm-season grasses cause hepatogenous photosensitization, among them several species of *Brachiaria.* Steroidal saponins present in these plants may be responsible for liver damage. However, sporidesmin and other hepatotoxic mycotoxins are likely to produce synergistic effects, which could explain the sporadic incidence of poisoning. The objective of this study was to evaluate the relation between steroidal saponin (measured by HPLC/ELSD), *Pithomyces chartarum* spores present in plots of *Brachiaria*, and the possible influence of meteorological factors on saponin and spores levels. The saponin detected in *B. brizantha* and *B. decumbens* was protodioscin. The saponin concentration was higher in immature plants during early growth; further, *B. decumbens* had a higher amount of saponin than found in *B. brizantha*. The level of saponin concentration was moderately influenced by weather variables; the two main variables were the maximum sunshine duration and total cumulative precipitation. *P. chartarum* spore count was higher in older plants and did not differ between *B. brizantha* and *B. decumbens*. Spore counting was influenced by maximum precipitation and average evaporation (Class A pan).

Keywords: protodioscin, sporidesmin, tropical grasses

Introduction

Brachiaria species are cultivated worldwide in tropical and subtropical climates due to their high productivity, and these grasses are critical for meat and milk production in many countries as the main forage source for ruminants (Miles et al. 1996).

The extraordinary reliance on *Brachiaria* in Brazil, as in other tropical countries, is justified by the advantages that this grass has over other species. *Brachiaria* spp. provide excellent yields of green matter in soils with low and medium fertility. They are drought tolerant, resistant to pests, and are well adapted to acidic and poor soils. These species are not only valuable plants for erosion control as a ground cover, but they also tolerate heavy grazing. Moreover, they have the ability to adapt to different environments; they are aggressive and compete with the native vegetation. Further, the seeds have resistivity which allows these plants to establish in, and dominate, native vegetation (Dias-Filho 2000, Argel et al. 2007, Hare et al. 2009).

Pithomyces chartarum (Berk & Curt) M. B. Ellis is a broad-based saprophytic fungus found in tropical, subtropical, and temperate regions. It is mostly found in dead plant material in native pastures, primarily those that are cultivated. Numerous conidia (spores) are produced under

favorable environmental conditions and contain the hepatotoxic sporidesmin, which can cause hepatogenous photosensitization. Experimental reproduction of the fungus indicates that most of the samples (isolates) from Australia and New Zealand produce sporidesmin (Di Menna et al. 2009).

Saponins are substances found in at least 400 plant species, many of which are used as food or supplements in ruminant nutrition. Saponins have beneficial effects as growth promoters, but they sometimes are toxic to ruminants. Saponins are derivatives of secondary plant metabolism, related to the plant defense system, and are mainly found in tissues that are most vulnerable to fungal or bacterial attack or insect predation (Wina et al. 2005). *Brachiaria* species and other plants (*Panicum* spp., *Tribulus terrestris*, and *Narthecium ossifragum*) contain steroidal saponins, which are associated with deposition of crystalloid material in the biliary system, cholangitis, and hepatogenous photosensitization (Driemeier et al. 2002, Brum et al. 2007, Fagliari et al. 2007, Uhlig et al. 2007, Botha and Penrithb 2008, Badiei et al. 2009, Lee et al. 2009, Assumaidaee et al. 2010, Saturnino et al. 2010).

The objective of this study was to evaluate the relation between steroidal saponins and *Pithomyces chartarum* spores present in plots of *Brachiaria* and the possible influence of meteorological aspects of the environment on concentration of saponins and spore counts.

Materials and Methods

Twenty-four plots measuring 3x4 m were planted, 12 with *B. decumbens* cv. Basilisk and the other 12 with *B. brizantha* cv. Marandu, at the Federal University of Goiás, Goiânia, Brazil (16°36'11,35"S 49°16'06,50"W; elevation 770 m). Each plot had 14 lines of plants; the outside lines were not harvested, and two inside lines were harvested at each sample period.

Nitrogen fertilization was applied after an initial soil analysis. Nitrogen was applied evenly to all plots at one of three levels (0, 50, 100 kg/hectare), 30 days after planting.

Plant development was followed up for 12 months. The plants were sampled on days 60, 120, 180, 240, 300, and 360 after planting to determine spore concentration and saponin concentration during this period. Plants were clipped near ground level (5cm), and the green material was collected to determine the quantity of spores and saponin concentration. Although the total plant was collected, the leaves and stems were subsequently separated and the analysis was only done on leaf material.

Spore Counting

The morphologic identification of the fungal spores from *Pithomyces chartarum* was based on Dingley´s (1962) description. The technique used for spore counting was adapted from Di Menna and Bailey (1973). Leaves were cut into 2 cm pieces. Sixty grams of fresh grass were mixed by hand in 600 mL of water. After 1 minute of agitation, spores were counted using a microscope counting chamber (hemocytometer). The quantities of spores counted in a $2mm^3$ are multiplied by 5,000 which provides the quantity of spores in 1g of forage.

Extraction and Isolation of Saponin

The leaves of *B. decumbens* and *B. brizantha* were dried at room temperature and ground in the Wiley mill (3 mm). The dried material was stored in airtight bags and analyzed about 6 months after each sampling.

Extraction of saponin

Dried powdered leaf samples were processed in duplicate. Each sample (1 g) was extracted three times in 50% acetonitrile and water (v/v) using volumes of 8 mL, 5 mL, and 3 mL under ultrasonic agitation for 30 min, 30 min, and 20 min, respectively. Next, the extracts were transferred to a test tube and centrifuged for 30 min at 5,000 rpm at 13ºC. The extracted solution was completed to 10 mL volume for analysis using HPLC/ELSD (high-performance liquid chromatography/evaporative light scattering).

HPLC/ELSD

Saponin was quantified using a modification of the technique of Ganzera et al. (2001). The analysis was done with a Shimadzu HPLC, model LC-10AD coupled to the detector. The evaporative light-scattering detector from Shimadzu model ELSD-LTs was used for quantitative analysis of protodioscin. The column used was a Shim-Pack CLC-ODS (4.6 x150 mm, 5.0 µm) using a gradient system with acetonitrile (B)/water (A), starting at 20% B for 5 min, 35% B for 12.5 min, and 20% B for 5 min; flow 1mL/min. The injection volume was 10 µL using a 10 µL loop.

Determination of Meteorological Parameters

Meteorological dates refer to the daily averages of each period (interval of 60 days). Data were

acquired in Goiânia, Goiás, Brazil, from December 2007 to December 2008 and provided by the meteorological station of the Federal University of Goiás, about 1 km from the plots.

Twenty-five variables were analyzed: the mean, maximum, and minimum of sunshine duration (Campbell-Stokes sunshine recorder); solar radiation; relative humidity; maximum temperature; minimum temperature; evaporation (class A pan); evaporation (Piche evaporimeter); and precipitation (mean, maximum, cumulative/period, and total cumulative). The equipment and measurements used adhered to the international standard (WMO 2008).

Statistical Analysis

Data for spores were tested for normality and were not normally distributed. The evaluation of the spores and saponin concentration between groups were assessed using the non-parametric Mann-Whitney U test with a significance level of 5% (Sampaio 2007). For the analysis within groups at each level of nitrogen, the Kruskal-Wallis test was used. The correlation between spore counts, saponin concentration, and meteorological parameters was examined using Pearson's correlation and multiple regression.

Correlation analysis and multiple regression procedures with influence diagnostics (e.g. residuals, examining outliers, and leverage) were used in SAS (2007) to determine their relationships. After this initial analysis, a stepwise multiple regression was used in an exploratory analysis to screen independent variables, followed by a hierarchical multiple regression with two variables introduced in a specific order for the final regression equations. Multi-co-linearity is loosely defined as a regression model in which two or more predictor variables are highly correlated; this greatly inflates the R^2 value. The Variance Inflation Factor (VIF) is a common method for detecting multi-co-linearity. VIF (the reciprocal of the tolerance) was examined for each model, and models with VIF greater than 4 were discarded (Belsley et al. 1980). A VIF of this magnitude indicates that the variance of the estimated coefficient is more than 4 times larger than it would be if the predictors were not correlated. The condition index was also used as another indicator of co-linearity; a condition index greater than 30 suggests that some variables are highly correlated, and the model should be refit (Yu 2000). The variables were discarded with a condition index greater than 15. Additionally, co-linearity was diagnosed by recognizing variables that had large proportions of variance (0.50 or more) that corresponded to large condition indices.

The best models were defined as those that had a high R^2, were significant at $P < 0.05$, and had a relatively small value of Mallow's *Cp* statistic, where *Cp* was approximately equal to the number of terms in the model. *Cp* tends to find the best subset of the model that includes only the important predictors of the dependent variable. The data were not compared for R^2 between periods and species because the variances of some of the independent variables were not equal.

Because the purpose of this study was to determine if there was a relationship between the weather and concentrations of saponin and the spore counts, there was no cross validation attempted in this study to forecast levels of saponin and spore count in other populations of plants.

Results

The saponin detected in *B. brizantha* and *B. decumbens* was protodioscin. The level of nitrogen fertilization did not influence ($P > 0.10$) the amount of spores or saponin concentration. For this reason the results were grouped by species of *Brachiaria*, independent of the level of fertilization (table 1 and figure 1).

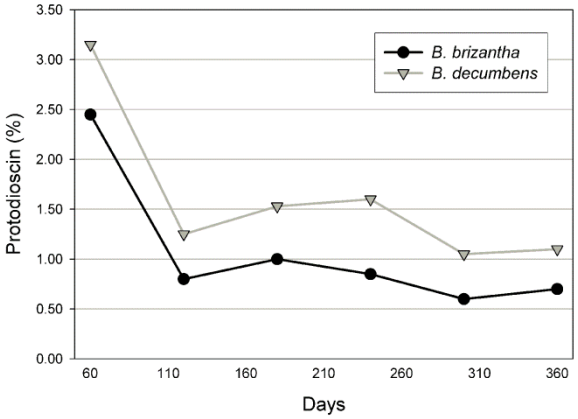

Figure 1. Average saponin concentration (% of dry weight) in *Brachiaria brizantha* and *Brachiaria decumbens*.

The level of saponin detected in *B. decumbens* was higher ($P < 0.05$) than in *B. brizantha* at all time periods. Both of the *Brachiaria* species had the highest average concentration of saponin during the first sampling period at 60 days ($P < 0.05$), then decreased around 70 percent at 120 days and the lowest level was observed at 300 days.

Table1. Saponin average concentration (%) and spore counts (*P. chartarum*) in *Brachiaria brizantha* (BB) *and Brachiaria decumbens* (BD)[1], shown by day of harvest after planting (day 0)

Day/Species	Saponin		Spores	
	BB	BD	BB	BD
60 days	2.39±0.30^{a}	3.15±0.52*^{a}	3,750±3,750^{a}	3,333±3,888^{a}
120 days	0.81±0.20^{bc}	1.26±0.37*^{b}	1,250±1,875^{a}	417±763^{a}
180 days	0.99±0.48^{ab}	1.56±0.50*^{b}	8,750±7,083^{abc}	7,083±4,236^{ab}
240 days	0.83±0.21^{bc}	1.65±0.28*^{ab}	5,833±3,750^{ab}	2,917±2,916^{a}
300 days	0.61±0.09^{c}	1.06±0.35*^{b}	39,167±19,166^{c}	36,250±26,458^{b}
360 days	0.70±0.21^{bc}	1.11±0.30*^{b}	24,167±13,472^{bc}	34,583±26,111^{b}

[1] BB = *B. brizantha*; BD = *B. decumbens*. Plants were sampled at the intervals shown by clipping leaves to near ground level.

[2] Analysis was done using the Kruskal-Wallis test. Asterisks in the same row indicate that the results between plant species differ at $P < 0.05$ *Brachiaria* (BB and BD). Different letters in the same column indicate that the results differ over time ($P < 0.05$) within each species of *Brachiaria*.

The amount of spores ranged from zero to 145,000 spores/g of forage. The spore count in older plants (300 and 360 days) was higher ($P < 0.05$) than in young plants (60 and 120 days) in both species. Several plots of both species had no spores at various time periods, with the exception of day 300, when all the plots had some spores (figure 2).

There was no correlation between the amount of saponin and spores over the periods analyzed ($R^2 = -0.26$). Weather variable means are presented in table 2 to show seasonal variation. The correlations between the main meteorological parameters and the amount of saponin and spores are shown in table 3.

Although the maximum sunshine duration and maximum precipitation were not highly correlated (table 2) with saponin concentration or spore count, these parameters were entered into the multiple regression models and both, with other variables, explained a substantial amount of the variation. Maximum hours of sunshine duration and total cumulative precipitation were related to saponin concentration (R^2=0.55; Cp=3.0) by:

$$y = -0.14 + 0.34\ (\textit{Sunshine Duration Max}) - 0.002\ (\textit{Precipitation Total Cumulative})$$

Maximum precipitation and mean evaporation (Class A pan) were the best 2-variable model (R^2=0.40; Cp=0.32) explaining a substantial portion of the variation associated with spore counts:

$$y = -70923 + 126.8\ (\textit{Precipitation Max}) + 14812\ (\textit{Evaporation Class A Pan Mean})$$

Discussion

For saponin analysis, we used high-performance liquid chromatography. Due to the polar nature of this compound, HPLC is usually chosen for quantitative analysis. The ELSD detector reduces the interference of impurities and the gradient elution of the mobile phase improves the accuracy for saponin determination (Yang et al. 2003).

In the present study, saponin concentrations decreased in both BB and BD plants as they matured. The results are similar to those of Castro et al. (2011). They studied the levels of protodioscin in different *Brachiaria* spp. at various stages of maturity, and they concluded that young plants in the growth phase have a higher amount of saponin than older plants. Castro et al. (2011) reported that protodioscin concentrations of 0.3% to 2.56% were sufficient to induce hepatogenous photosensitization when fed to sheep.

Additionally, *B. decumbens* had higher levels of saponin than *B. brizantha*, which was also reported by Lee et al. (2011). The steroidal saponin protodioscin identified in this study has been described in many plants: *Dioscoera gracillima*, *Melilotus tauricus*, *Dioscoera colletti*, *Asparagus officinalis* L., *Smilax china* L., *Costus speciosus* (Ahmad and Basha 2006), *Panicum virgatum* L. (Lee et al. 2009), and especially in *Brachiaria* spp. (Riet-Correa et al. 2011).

Ferreira et al. (2011) studied the amount of protodioscin in *B. brizantha* as influenced by various levels of maturity, and examined the relationship between saponin, sunshine duration, temperature, and relative humidity. They found that young leaves

Table 2. Weather variables (average per 60-day interval) measured at the Federal University of Goiás, Goiânia, Brazil, near the *Brachiaria* spp. plots. Days correspond to periodic harvesting of *Brachiaria* spp. forage after planting from cultivated plots

Variables	Days	60	120	180	240	300	360
	Months	Dec-Feb	Feb-Apr	Apr-Jun	Jun-Aug	Aug-Oct	Oct-Dec
	Seasons	Summer	Autumn	Autumn	Winter	Winter/ Spring	Spring
Sunshine Duration (hours/Day)	Mean	4.3	5.3	7.9	8.9	8.1	5.6
	Max	9.9	9.7	9.8	9.8	10.2	10.7
	Min	0.0	0.0	0.0	6.2	0.0	0.0
Solar Radiation (MJ/m²)	Mean	17.2	16.8	15.8	17.6	22.5	17.9
	Max	28.9	22.6	20.2	21.2	29.1	29.8
	Min	1.3	8.8	4.1	12.2	5.8	1.6
Relative Humidity (%)	Mean	85.2	85.3	72.0	63.1	57.9	69.5
	Max	95.0	95.0	91.0	73.0	93.0	84.0
	Min	75.0	76.0	54.0	54.0	41.0	53.0
Precipitation (mm)	Mean	9.1	8.9	3.3	0.0	1.6	5.4
	Max	42.0	56.4	100.7	0.0	36.4	80.8
	Min	0.0	0.0	0.0	0.0	0.0	0.0
	Cumulative/ Period	437	527	196	0	97	324
	Total Cumulative	437	963	1159	1159	1256	1580
Max Temperature (°C)	Mean	29.6	30.1	29.7	30.4	32.9	31.6
	Max	33.4	34.2	32.4	34.6	37.2	37.8
	Min	23.8	23.6	20.7	23.2	22.2	26.2
Min Temperature (°C)	Mean	19.1	19.1	14.3	10.2	14.5	19.1
	Max	20.8	20.4	20.3	14.8	21.8	21.8
	Min	17.4	17.0	9.2	5.8	7.8	14.8
Evaporation – Class A pan (mm)	Mean	4.8	4.3	4.5	5.0	7.0	6.1
	Max	9.6	9.8	9.7	8.9	10.6	10.0
	Min	0.7	0.5	0.2	0.9	2.1	2.1
Evaporation – Piche evaporimeter (mm)	Mean	2.7	2.4	1.7	3.2	3.9	2.8
	Max	4.7	6.2	4.2	9.3	18.5	8.0
	Min	0.8	0.1	0.0	1.0	0.1	0.2

Table 3. Pearson correlation between weather variables, saponin concentration, and spore counts for *Brachiaria* forage harvested every 60 days after planting at the Federal University of Goiás, Goiânia, Brazil

	Saponin		Spores	
Variable	r	P	r	P
Sunshine Duration Mean	-0.42	<0.0001	0.10	0.2377
Sunshine Duration Max	0.12	0.1410	0.22	0.0097
Relative Humidity Mean	0.37	<0.0001	-0.43	<0.0001
Relative Humidity Min	0.50	<0.0001	-0.41	<0.0001
Precipitation Max	0.06	0.4502	0.08	0.3288
Precipitation Total Cumulative	-0.56	<0.0001	0.40	<0.0001
Max Temperature Mean	-0.51	<0.0001	0.55	<0.0001
Max Temperature Max	-0.24	0.004	0.49	<0.0001
Evaporation (Class A pan) Mean	-0.37	<0.0001	0.50	<0.0001
Evaporation (Class A pan) Max	0.06	0.4544	0.28	0.0008
Evaporation (Class A pan) Min	-0.38	<0.0001	0.54	<0.0001
Evaporation (Piche) Mean	-0.23	0.0047	0.34	<0.0001
Evaporation (Piche) Max	-0.34	<0.0001	0.49	<0.0001
Evaporation (Piche) Min	0.44	<0.0001	-0.25	0.0025

had higher levels of protodioscin ($P < 0.01$) than did mature and old leaves (3.61±1.12%, 1.94±0.97%, and 1.01±0.79%, respectively); saponin variation in young leaves was related ($r = 0.9$; $P < 0.05$) to sunshine duration and in mature leaves with Meteorological data have suggested that higher levels of saponins in *Brachiaria* spp. are found during periods with greater rainfall (Barbosa et al. 2006, Moreira et al. 2009). In the present study, the duration of sunshine and maximum ambient temperature showed a negative correlation ($r = -0.42$ and $r = -0.51$, respectively) with saponin and a positive correlation with minimum relative humidity mean ($r = 0.50$). There was a negative correlation between saponin concentration and total cumulative precipitation ($r = -0.56$). The harvest at 60 days with high precipitation (437 mm) had the highest percentage of saponin. However, the same result was not repeated at the harvest of 120 days (527mm) and 360 days (324mm) when precipitation was also high. These results suggest that age of the plant is the major influence on saponin levels and not precipitation. These results also suggest that saponin concentrations may be a complex interaction involving several factors, including plant age, geographic location, and cultivation conditions (Yang et al. 2003).

Contrary to our results, Brum et al. (2009) evaluated saponins by a quantitative method (thin layer chromatography and spectrophotometric analysis) and found larger quantities of protodioscin in mature *Brachiaria* during seed shatter. The range of saponin concentration in *B. brizantha* was 0.53% to 2.09%, greater than that found in *B. decumbens* at 0.8% to 1.9%. Outbreaks of hepatogenous photosensitization were evaluated in Pará State, Brazil, where Albernaz et al. (2010) and Silveira et al. (2009) found toxic levels of protodioscin for sheep and goats grazing on *B. brizantha* in an advanced state of maturity during drought period. Saturnino et al. (2010) did not measure the levels of saponin but found that mature *B. decumbens* during seed shatter poisoned sheep.

The toxic dose of the saponin protodioscin from *Brachiaria* spp. for ruminants has not been clearly established (Brum et al. 2009). Outbreaks of hepatogenous photosensitization were detected in sheep that were fed *Brachiaria* containing 0.3% to 2.56% protodioscin (Castro et al. 2011), 0.88% protodioscin (Albernaz et al. 2010), 2.36% protodioscin (Brum et al. 2007), and for goats 1.54% protodioscin (Silveira et al. 2009). However, cattle fed with *Brachiaria* containing 1.09% (Moreira et al. 2009) and 1.63% (Brum et al. 2007) protodioscin did not develop clinical signs of disease.

The ideal conditions for *Pithomyces chartarum* growth have been described as pastures with old leaves and mulch, relative humidity 60%, and ambient temperature from 18 to 27°C. However, the production of sporidesmin is optimal when relative humidity remains at 100 percent and temperature remains around 20 to 25°C. These conditions occur when the rainy season is followed by a dry, hot period, which is ideal for pasture growth (Brook 1964, Bars et al. 1990, Alvariza 1993, Russomanno et al. 2003, Di Menna et al. 2009). This study shows that the periods of grass growth of 300 and 360 days had ideal conditions for fungal growth with plenty of old leaves and decaying material, high temperatures, precipitation, and evaporation. During these periods, the amount of spores was significantly higher ($P < 0.05$) compared with earlier periods with young plants (60 and 120 days).

Moreira et al. (2009) evaluated the spore counts in *Brachiaria* spp. in Goiás State, Brazil, where cattle showed no clinical signs of the disease and observed that in the dry season, counts ranged from 5,000 to 40,000 spores/g of pasture, and during the rainy season the score was higher, up to 50,000 spores/g of pasture. However, pastures throughout these experiments attained a maximum height of 6 cm and also did not have old leaves, conditions that did not favor the growth of the fungus.

Clinical and sub-clinical lesions were observed in cattle grazing on *Brachiaria* spp. containing 0 to 15,000 spores/g of pasture (Fioravanti 1999). The absence of spores or low spore counts do not exclude the fungus as the etiologic agent of sporidesmin toxicosis because there is a lag in the time between when spores appear and another lag from the time of ingestion of sporidesmin and appearance of lesions (Smith 2000, Di Menna et al. 2009).

The sporidesmin detected in plant material corresponded to the number of spores found in the sample in data from Oceania. However, this relationship is not absolute and discrepancies have been reported (Collin et al. 1995, Di Menna et al. 2009).

The fungus *P. chartarum* normally produces sporidesmin but some strains do not produce sporidesmin. Most researchers agree that the isolates from New Zealand and Australia produce more sporidesmin than those from the Americas. Lemos et al. (1996) examined 30 isolated cases of *P. chartarum* from *B. decumbens* with a history of outbreaks in sheep in Brazil, and only one isolate

produced sporidesmin. They concluded that the disease was caused by *B. decumbens* and not by the toxin from the fungus. Collin et al. (1998) evaluated 51 isolates of *P. chartarum*, including 9 samples from Brazil. Only a single Brazilian sample produced sporidesmin (2%) while isolates from New Zealand produced sporidesmin (86% of 391 samples), as did those from Australia (67% of 207) and Uruguay (28% of 182). Halder et al. (1981) analyzed samples from North America and did not observe the production of sporidesmin. DNA analysis proved that the strains producing sporidesmin from New Zealand are different than those non-producing strains from South Africa and the Americas (Beever and Parkes 1993).

Until now the small number of isolates studied from Brazil is not sufficient to conclude that the strains of Brazil do not produce sporidesmin. Future studies should be developed with Brazilian samples to determine conclusively if the type and amount of fungal growth produces sporidesmin. The interaction of saponin type and concentration and toxicity in ruminants also requires further investigation.

Conclusions

The saponin found in *B. brizantha* and *B. decumbens* in Goiás, Brazil, was protodioscin. The saponin concentration was higher in immature plants during early growth; further, *B. decumbens* had a higher concentration of saponin than *B. brizantha*. The most important weather variables that influenced the saponin concentration were maximum duration of sunshine and total cumulative precipitation.

The *P. chartarum* spore count was higher in older plants and did not differ between *B. brizantha* and *B. decumbens*. Spore counting was influenced primarily by maximum precipitation and average evaporation (Class A pan).

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Veratrum-Induced Placental Dysplasia in Sheep

Kevin D. Welch*, Kip E. Panter, Bryan L. Stegelmeier, Stephen T. Lee, Dale R. Gardner, and Daniel Cook

USDA-ARS Poisonous Plant Research Laboratory, Logan, UT

*Corresponding author: Kevin Welch, kevin.welch@ars.usda.gov

Abstract

Cyclopamine, a steroidal alkaloid from *Veratrum californicum*, is teratogenic causing a range of birth defects including cyclopia (synophthalmia) as well as other craniofacial and structural malformations. Previous studies have indicated that fetuses with cyclopia are smaller, under developed, and appear premature compared to gestational matched normal fetuses. Preliminary observations suggest this could be due to placental dysplasia. The objective of this study was to determine if there are placental dysplasias in ewes with fetuses with synophthalmia and other less severe craniofacial malformations. Ewes were dosed orally twice on gestation day (GD) 14 with 0.88 g/kg of dried *V. californicum*. Pregnancy, pre-partum fetal malformations, and placentome diameter were determined by ultrasound imaging on GD 45, 60, 75, 105, and 135. At GD 135 the ewes were euthanized and the fetuses were assessed for gross malformations and several fetal measurements were made as well as several measurements in the ewe. There was no difference between the controls and the treated animals in the placentome diameter measurements made in utero by ultrasound imaging. The 23 treated ewes were carrying 26 fetuses. Eleven of the fetuses were cyclopic, nine had other craniofacial malformations including maxillary dysplasia, mandibular micrognathia, and superior deviation of the rostral mandible, while six fetuses appeared normal. Cyclopic fetuses were smaller than fetuses with less severe craniofacial malformations, which were similar in size to normal fetuses. The number of placentomes, placentome area, and placentome weight were all significantly smaller for ewes with cyclopic fetuses. There was no difference in the size or weight of the fetal pituitary glands between the different groups of fetuses. However, weights of the fetal adrenal glands were significantly less in the cyclopic fetuses. In summary, there appears to be a correlation between the severity of the malformed fetuses and placental dysplasia.

Abbreviation

GD, Gestational Day

Keywords: cyclopamine, cyclopia, holoprosencephaly, sheep, synophthalmia, *Veratrum californicum*

Introduction

Cyclopia and a number of other teratogenic malformations occur in lambs when pregnant ewes graze *Veratrum californicum* early in gestation (Binns et al. 1962, Binns et al. 1965, Keeler et al. 1985, Keeler and Stuart 1987, Keeler 1990). Incidences as high as 25 percent were reported in flocks of 5,000-10,000 range ewes (Binns et al. 1963). Early evaluation of the chronology of teratogenicity of *V. californicum* in sheep indicated that gestation day (GD) 14 is the critical day for synophthalmia malformations to occur (Binns et al. 1965). Early embryonic death and resorption were

later associated with low reproductive rates when sheep were grazed in areas with abundant *V. californicum* (Binns et al. 1963, Van Kampen et al. 1969). The alkaloids responsible for terata induction in *V. californicum* have been identified as jervine, 11-deoxojervine (renamed cyclopamine), and cycloposine (the glycoside of cyclopamine) (Keeler and Binns 1968). The mechanism of cyclopamine-induced birth defects has been shown to result from the inhibition of the Sonic Hedgehog signal transduction pathway (Cooper et al. 1998, Incardona et al. 1998). The Hedgehog signaling pathway plays an integral role in cell growth and differentiation, including embryonic development of the eyes and maxilla (Rubenstein and Beachy 1998, Lum and Beachy 2004).

Previous studies demonstrated that fetuses with cyclopia were smaller, under developed, and appeared premature compared to normal fetuses of similar gestation age (Welch et al. 2009). Preliminary observations suggested this could be due to placental dysplasia (unpublished observations). The objective of this study was to determine if there are placental dysplasias in ewes with fetuses with synophthalmia and other less severe craniofacial malformations.

Materials and Methods

Plant Material

Root material from *V. californicum* plants was used as the source of cyclopamine for the oral dosing experiments in this study. It has been demonstrated that both the aerial and root/rhizome portions of the plant contain the teratogen cyclopamine (Keeler and Binns 1966a), and that both can induce "monkey faced lamb" defects (Binns et al. 1965). However, the concentration of cyclopamine is 5-10 times higher in root material (Keeler and Binns 1966a, 1966b, 1971). The plant material was collected in Muldoon Canyon at the headwaters of the Lost River Drainage in Idaho. Plant material was transported to our laboratory, air dried in sunlight, finely chopped, and stored in an enclosed shed at ambient temperature. Extraction of *V. californicum* for cyclopamine analysis was accomplished as described previously (Welch et al. 2009).

Animal Studies

Twenty-seven western white-faced ewes weighing 78 ± 11 kg were synchronized in estrus using intravaginal sponges impregnated with fluorogestone acetate (Intervet International B.V., Netherlands). Each ewe was hand mated to Suffolk rams 3 times a day for 3 days following removal of the intravaginal sponges; the last day that each ewe exhibited standing estrus was considered day 0 of gestation (Keeler and Stuart 1987, Keeler and Baker 1989, Jainudeen et al. 2000). Each ewe was dosed at 7 a.m. and 3 p.m. on GD 14 with ground plant material in order to limit maternal toxic effects of *V. californicum*. Twenty-three ewes were dosed with 0.88 g *V. californicum*/kg BW, and four ewes were dosed with 0.88 g alfalfa/kg BW as controls.

Ewes were evaluated via ultrasound imaging for pregnancy on GD 30. Pregnancy, pre-partum fetal malformations, and placentome diameter were determined by ultrasound imaging on GD 45, 60, 75, 105, and 135. The ewes were examined transabdominally using an Aloka SSD-900V scanner fitted with a 5 MHz convex electronic transducer (Wallingford, CT). The ewes were restrained on their backs to facilitate access to the hairless areas of the abdominal wall just in front of the udder. All ewes, including controls, were euthanized on GD 135, and the fetuses were assessed for gross malformations, and several fetal measurements were made as well as several placental measurements as listed in tables 2-5. All uterine and placental measurements are reported once for each ewe for each comparison and not for each fetus. Normal gestation for these sheep is approximately 150 days.

Analysis and Statistics

Statistical comparisons between two groups were performed using a Student's *T*-test and between three or more groups using ANOVA with a Bonferroni post hoc test of significance between individual groups as pairwise comparisons. Differences were considered significant at $P < 0.05$.

Results

Of the four ewes treated with alfalfa on GD 14, one was found to not be pregnant on GD 30 (table 1). The remaining three ewes each had normal fetuses (five fetuses total) at GD 135. Twenty-three ewes were treated with *V. californicum* on GD 14. Three of the 23 ewes were not pregnant on GD 30 (table 1). Five of the remaining 20 treated ewes were not pregnant on GD 135, indicating that embryonic/fetal death had occurred in these ewes. Twenty-six fetuses were found in the remaining 15 ewes. Eleven of those fetuses had cyclopia (synophthalmia), nine had other less severe craniofacial malformations (which are referred to as monkey-faced fetuses), and six were normal. In this study, we use the term "cyclopia" to refer to fetuses with a single eye

Table 1. Summary of the effects of *Veratrum* treatment on embryonic loss, birth defects, and number of fetuses

Category	Number of ewes	Number of fetuses
Breeding results		
Control ewes bred	4	
Control ewes not pregnant on GD 30	1	
Control ewes pregnant on GD 135	3	5
Treated ewes bred	23	
Treated ewes not pregnant on GD 30	3	
Treated ewes pregnant on GD 135	15	26
Treated ewes with confirmed loss of embryo after GD 30	5	
Type of birth defect		
Cyclops fetus	8	11
Monkey-faced fetus	6	9
Normal fetus	7	11
Normal fetus from treated ewe	4	6
Normal fetus from control ewe	3	5
Mix of normal & monkey-faced fetus	1	2
Mix of cyclops & monkey-faced fetus	2	4
Number of fetuses		
Single	7	7
Normal		2
Monkey-faced fetus		1
Cyclops fetus		4
Twins	9	18
Normal		9
Monkey-faced fetus		5
Cyclops fetus		4
Triplets	2	6
Monkey-faced fetus		3
Cyclops fetus		3

Figure 1. Craniofacial defects in fetuses associated with maternal ingestion of *V. californicum.*

socket. All other fetuses with less severe craniofacial malformations that have two eye sockets are referred to as monkey-faced fetuses. Various representations of the craniofacial malformations are represented in figure 1.

Seven ewes had single fetuses, two normal fetuses (one from a treated ewe and one from a control ewe), one monkey-faced fetus, and four cyclopic fetuses (table 1). Nine ewes had twin fetuses, nine normal fetuses (four from control ewes and five from treated ewes), five monkey-faced fetuses, and four cyclopic fetuses. Two ewes had triplet fetuses, three monkey-faced fetuses and three cyclopic fetuses. Each set of triplet fetuses were the same, i.e., all cyclopic or all monkey-faced. However, three of the sets of twins were mixed, one set of normal and monkey-faced fetuses and two sets of cyclopic and monkey-faced fetuses.

Ultrasound imaging was used to measure the in utero placentome diameter on GD 45, 60, 75, 105, and 135 (figure 2). Measurements were recorded for each ewe throughout the experiment. Once the experiment was over, the ewes were then categorized into either treated or control, depending upon their treatment. The ewes were also categorized according to the malformations that their fetuses had, i.e., normal, monkey-faced, or cyclopic. A statistical comparison of the in utero placentome diameters demonstrated no difference between control and treated ewes ($P = 0.16$, figure 2A), and there was no difference amongst three outcome groups ($P = 0.19$, figure 2B). In both comparisons, there was a day effect ($P < 0.001$), as the placentomes were shown to increase in size from GD 45 to GD 60. However, there was no group x day effect for either comparison ($P = 0.27$ and $P = 0.41$).

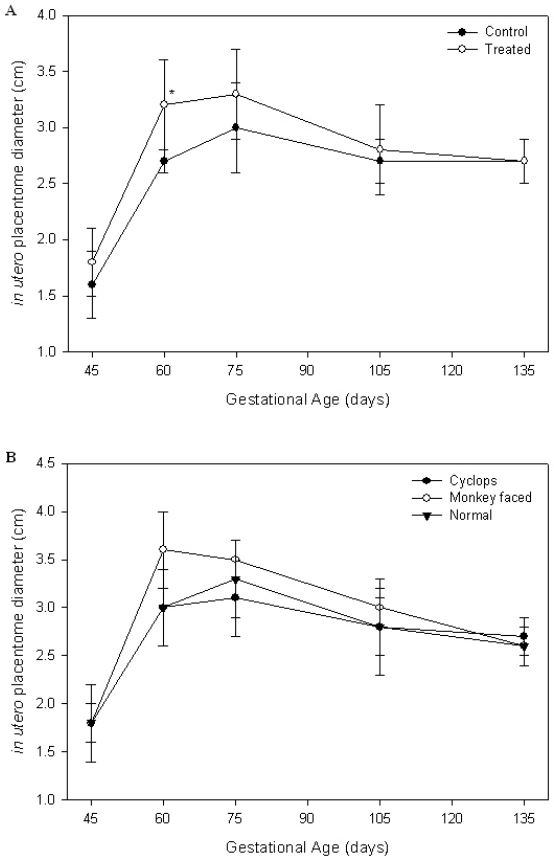

Figure 2. Diameter of placentomes measured *in utero* via ultrasound imaging. Results represent the mean ± SD of 3 to 15 ewes per group. (A) Control versus treated ewes; (B) ewes with cyclops, monkey-faced, or normal lambs. * $P < 0.05$ as compared to controls.

At GD 135 all ewes were euthanized and their gravid uterus was removed. Numerous maternal and fetal measurements were made in order to determine if either *Veratrum* treatment or the type of fetus was correlated with the development of placentation. Due to the complexity and potential confounding factors, four different comparisons were performed: (1) comparison of treatment, (2) comparison of the type of fetuses, (3) comparison of the type of twin fetuses, and (4) comparison of the number of fetuses. First, the measurements from normal fetuses from treated ewes were compared to the measurements from the normal fetuses from control ewes to determine if *Veratrum* treatment had any effect (table 2). The only difference observed was that the fetuses from treated ewes had significantly larger adrenal glands as measured by weight ($P = 0.001$). Therefore, for the remaining comparisons, the normal fetus data for the treated and control ewes were combined in order to increase the sample size for statistical power.

The comparison of the measurements between the types of fetus demonstrated that there was a difference in the number of placentomes and the placentome weight between ewes with normal and cyclopic fetuses, with ewes with normal fetuses having more placentomes that were also larger (table 3). The cyclopic fetuses were found to be significantly smaller than both the monkey-faced and normal fetuses, demonstrated by the lower fetal weight and smaller crown to rump length, abdominal and thoracic circumference, as well as smaller fetal brain and adrenal weights. Of note, a comparison of the cyclopic fetus fetal adrenal weights compared to only the fetal adrenal weight from fetuses born to control ewes is still statistically significant ($P < 0.001$). Similarly, the monkey-faced fetuses were also found to be smaller than the normal fetuses. In general, similar results were observed when restricting the comparison of the types of fetuses to only fetuses that were part of a set of twins (table 4). Notable exceptions were the fact that the gravid uterus weight for ewes with cyclopic fetuses was less than that of ewes with normal fetuses. Even though ewes with twin normal fetuses had more placentomes than ewes with twin cyclopic fetuses, there was no difference in the average placentome weights.

A comparison of the measurements when categorized according to the number of fetuses from each ewe found only differences that would be expected with increasing number of fetuses in each gravid uterus, i.e., the gravid uterus weight, empty uterus weight, as well as the allantoic and amniotic fluid volumes were larger in ewes with multiple fetuses (table 5). The one additional difference observed was single fetuses had smaller average placentome weights than twin fetuses.

Discussion

Early embryonic development and maternal recognition of pregnancy require numerous processes to occur at specific times as well as specific hormonal balances in the uterine environment (DeSesso 2006). This environment is created by both the embryo and the uterus (Ashworth and Bazer 1989). The importance of environment and timing is highlighted by the fact that approximately 30 percent of the embryos are lost during early development (Dixon et al. 2007). The uterus exerts its own influence on embryonic development through histotrophic nutrition (Bazer et

Table 2. A comparison of normal fetuses from control ewes and normal fetuses from treated ewes

Measurement	Treated ewes			Control ewes		
	AVG	SD	n	AVG	SD	n
Gravid uterus weight (kg)	14.6	± 3.2	4	12.0	± 3.2	3
Empty uterus weight (kg)	2.5	± 0.4	4	2.3	± 0.3	3
Allantoic & amniotic fluid volume (l)	2.3	± 0.8	4	1.8	± 0.7	3
Number of placentomes	83.0	± 17.7	4	91.7	± 3.1	3
Placentome area (cm^2)	13.3	± 3.4	4	7.9	± 1.3	3
Placentome weight (g)	14.8	± 1.6	4	12.5	± 2.7	3
Fetal weight (kg)	5.6	± 0.8	6	4.9	± 0.4	5
Crown rump length (cm)	51.6	± 4.0	6	50.5	± 4.7	5
Abdominal circumference (cm)	33.2	± 2.6	6	35.7	± 2.2	5
Thoracic circumference (cm)	35.8	± 1.7	6	35.9	± 1.3	5
Fetal brain weight (g)	56.9	± 4.8	6	53.6	± 3.6	5
Fetal pituitary weight (g)	0.5	± 0.3	6	0.2	± 0.0	5
Fetal adrenal weight (g)	0.6	± 0.1*	6	0.4	± 0.1	5
Fetal trachea diameter (cm)	1.1	± 0.5	6	0.7	± 0.1	5

* = different from control at $P < 0.05$

Table 3. Comparison of measurements between fetuses with different defects

Measurement	Cyclops fetuses		Monkey-faced fetuses		Normal fetuses	
	AVG±SD	n	AVG±SD	n	AVG±SD	n
Gravid uterus weight (kg)	9.2 ± 2.8	8	13.9 ± 5.8	6	13.5 ± 3.2	7
Empty uterus weight (kg)	1.8 ± 0.5	8	2.2 ± 0.7	6	2.4 ± 0.4	7
Allantoic & amnionic fluid volume (l)	3.4 ± 1.2	8	3.5 ± 1.4	6	2.1 ± 0.7	7
Number of placentomes	59.1 ± 21.6 [n]	8	76.5 ± 22.0	6	86.7 ± 13.5 [c]	7
Placentome area (cm^2)	7.7 ± 1.4	8	8.8 ± 1.8	6	10.9 ± 3.8	7
Placentome weight (g)	9.5 ± 2.5 [n]	8	12.0 ± 3.2	6	13.8 ± 2.3 [c]	7
Fetal weight (kg)	2.3 ± 0.5 [m,n]	11	4.2 ± 0.9 [c,n]	9	5.3 ± 0.7 [c,m]	11
Crown rump length (cm)	41.3 ± 4.1 [m,n]	11	49.0 ± 3.6 [c]	8	51.1 ± 4.2 [c]	11
Abdominal circumference (cm)	27.1 ± 2.6 [n]	11	28.4 ± 2.5 [n]	8	34.3 ± 2.6 [c,m]	11
Thoracic circumference (cm)	27.0 ± 1.4 [m,n]	11	30.9 ± 2.2 [c,n]	8	35.9 ± 1.5 [c,m]	11
Fetal brain weight (g)	13.5 ± 8.6 [m,n]	11	33.5 ± 10.4 [c,n]	8	54.2 ± 4.7 [c,m]	11
Fetal pituitary weight (g)	0.3 ± 0.2	11	0.2 ± 0.2	7	0.3 ± 0.3	11
Fetal adrenal weight (g)	0.2 ± 0.1 [m,n]	11	1.5 ± 0.3 [c]	9	0.6 ± 0.1 [c]	11
Fetal trachea diameter (cm)	0.5 ± 0.3	11	0.9 ± 0.5	9	0.9 ± 0.4	11

[c] = different from cyclops fetuses at $P < 0.05$
[m] = different from monkey-faced fetuses at $P < 0.05$
[n] = different from normal fetuses at $P < 0.05$

Table 4. Comparison of measurements between twin fetuses with different defects

Measurement	Cyclops fetuses		Monkey-faced fetuses		Normal fetuses	
	AVG±SD	n	AVG±SD	n	AVG±SD	n
Gravid uterus weight (kg)	11.0 ± 1.1 [n]	3	13.0 ± 2.8	4	15.2 ± 1.3 [c]	5
Empty uterus weight (kg)	2.0 ± 0.4	3	2.2 ± 0.5	4	2.6 ± 0.2	5
Allantoic & amnionic fluid volume (l)	3.2 ± 0.8	3	2.7 ± 0.6	4	2.5 ± 0.5	5
Number of placentomes	56.0 ± 15.7 [n]	3	77.3 ± 23.7	4	91.8 ± 3.8 [c]	5
Placentome area (cm^2)	9.1 ± 0.3	3	8.9 ± 0.5	4	10.9 ± 3.7	5
Placentome weight (g)	12.3 ± 0.3	3	12.5 ± 1.4	4	14.5 ± 2.3	5
Fetal weight (kg)	2.6 ± 0.4 [m,n]	4	4.4 ± 0.7 [c]	5	5.1 ± 0.7 [c]	9
Crown rump length (cm)	44.1 ± 5.0 [m,n]	4	50.1 ± 1.6 [c]	4	49.7 ± 2.9 [c]	9
Abdominal circumference (cm)	25.6 ± 2.9 [n]	4	28.7 ± 3.6 [n]	4	34.0 ± 2.6 [c,m]	9
Thoracic circumference (cm)	27.5 ± 1.4 [m,n]	4	31.1 ± 2.2 [c,n]	4	35.7 ± 1.5 [c,m]	9
Fetal brain weight (g)	21.3 ± 3.2 [m,n]	4	35.2 ± 11.7 [c,n]	5	54.5 ± 5.0 [c,m]	9
Fetal pituitary weight (g)	0.3 ± 0.1	3	0.3 ± 0.1	4	0.3 ± 0.3	9
Fetal adrenal weight (g)	0.2 ± 0.1 [n]	4	0.4 ± 0.2	5	0.6 ± 0.1 [c]	9
Fetal trachea diameter (cm)	0.8 ± 0.4	4	0.8 ± 0.5	5	0.9 ± 0.4	9

[c] = different from cyclops fetuses at $P < 0.05$
[m] = different from monkey-faced fetuses at $P < 0.05$
[n] = different from normal fetuses at $P < 0.05$

Table 5. Comparison of measurements between single, twin, and triplet fetuses

Measurement	Single fetus		Twin fetuses		Triplet fetuses	
	AVG±SD	n	AVG±SD	n	AVG±SD	n
Gravid uterus weight (kg)	7.6 ± 1.6 [2,3]	7	13.8 ± 2.4 [1]	9	15.2 ± 7.6 [1]	2
Empty uterus weight (kg)	1.6 ± 0.4 [2,3]	7	2.4 ± 0.4 [1]	9	2.6 ± 0.3 [1]	2
Allantoic & amnionic fluid volume (l)	2.8 ± 1.5 [3]	7	2.7 ± 0.7 [3]	9	2.5 ± 0.9 [1,2]	2
Number of placentomes	58.6 ± 19.0	7	80.1 ± 19.9	9	91.8 ± 3.5	2
Placentome area (cm^2)	7.9 ± 3.4	7	10.0 ± 2.8	9	10.9 ± 2.8	2
Placentome weight (g)	8.5 ± 2.8 [2]	7	13.4 ± 1.8 [1]	9	14.5 ± 4.0	2
Fetal weight (kg)	3.5 ± 1.7	7	4.3 ± 1.2	18	5.1 ± 1.7	6
Crown rump length (cm)	45.7 ± 8.5	7	48.4 ± 3.9	17	49.7 ± 2.9	6
Abdominal circumference (cm)	30.4 ± 4.1	7	30.8 ± 4.6	17	34.0 ± 2.6	6
Thoracic circumference (cm)	30.3 ± 4.6	7	32.7 ± 3.8	17	35.7 ± 1.5	6
Fetal brain weight (g)	22.3 ± 22.2	7	41.8 ± 15.6	18	54.5 ± 5.0	5
Fetal pituitary weight (g)	0.3 ± 0.3	4	0.3 ± 0.2	16	0.3 ± 0.3	4
Fetal adrenal weight (g)	0.3 ± 0.2	7	0.4 ± 0.2	18	0.6 ± 0.1	6
Fetal trachea diameter (cm)	0.6 ± 0.4	7	0.8 ± 0.4	18	0.9 ± 0.4	6

[1] = different from single fetuses at $P < 0.05$
[2] = different from twin fetuses at $P < 0.05$
[3] = different from triplet fetuses at $P < 0.05$

al. 1993). In addition to uterine secretions, key embryonic secretions are also required for a synchronous interaction between uterine endometrium and embryonic tissues (Koch et al. 2010). Between GD 8-16 the developing conceptus secretes interferon-τ, which is thought to initiate the process of maternal recognition of pregnancy and is required for normal embryonic development (Spencer et al. 2004). Other studies have identified embryonic proteins associated with early embryonic attachment (Lee et al. 1998). Consequently, any alteration to the uterine environment during the early stages of development of the embryo can affect the ability of the embryo to develop normally, survive, and undergo normal parturition. A number of compounds that are teratogenic, due to their ability to alter the uterine environment or normal embryonic development, are plant toxins (Keeler 1984, Panter et al. 2011). In early studies of *Veratrum*-induced malformations, the observation was made that ewes with severely deformed fetuses had significantly prolonged gestations as a part of the syndrome (Binns et al. 1964, Van Kampen and Ellis 1972). In sheep, parturition is initiated by increased fetal hypothalamic-pituitary-adrenal (HPA) axis activity leading to fetal and maternal prostaglandin production and a rise in the maternal estradiol-progesterone (E2/P4) ratio (Kumarasamy et al. 2005). Estrogen up-regulates the expression of maternal endometrial prostaglandins, which stimulates myometrial contractility and labor ensues (Whittle et al. 2000). Corticotrophin releasing hormone (CRH) can stimulate the fetal release of ACTH to produce a cortisol surge, which leads to the onset of parturition, whereas inhibition of these processes can delay the onset of parturition (Chan et al. 1998). The placenta also plays an important role in normal hormone production, including growth hormone (Handwerger and Freemark 2000). Consequently, it is possible that veratrum treatment is altering normal HPA and/or placental function, resulting in delayed parturition and diminished fetal growth.

The observation was made in a recent study that a set of twin fetuses from a ewe treated with *Veratrum* appeared to be at different stages of development (Welch et al. 2009). A monkey-faced fetus was of normal size and fully covered with wool, similar to normal fetuses. However, its cyclopic twin was approximately two-thirds the size and had no wool. Although it is not uncommon to have normal twin fetuses of differing size, the difference in wool covering of these two twins is very unusual. Interestingly, in the same study, cyclopic fetuses that were surgically removed on GD 200 (normal gestation is 150 days) were of normal size and were fully covered in wool. Taken together, these observations indicate that the development of the cyclopic fetuses is hindered, and therefore more time is required for the fetus to reach normal weight and have normal wool covering. A potential, and possibly related, explanation could be deficiency of the placentation supporting cyclopic fetuses. The observation was made that the placentas from the ewes that delivered at GD 200 were not normal as the placentomes appeared smaller. Unfortunately, no assessments of the placentomes were made from the ewes that had delivered normally around GD 150. Consequently, the objective of this study was to determine if there are placental dysplasias in ewes with fetuses with synophthalmia and other less

severe craniofacial malformations versus ewes with normal fetuses.

There is evidence that placental growth processes during mid-gestation are critical because placental size determined during this period may have important repercussions during late gestation for functional capacity of the placenta to deliver nutrients to the fetus (Mellor 1983, Bell et al. 1987). In normal pregnancy and fetal development, the placental component exhibits a rapid increase in tissue weight until GD 75-80 and then declines (Ehrhardt and Bell 1995). The endometerium and myometrium steadily increase in weight from GD40-100, more than doubling in weight (Ehrhardt and Bell 1995). In this study, we found no difference in the uterine weight from a ewe with a cyclopic fetus versus a ewe with a normal fetus. However, the average placentome weights in ewes with cyclopic fetuses were lower than ewes with normal fetuses. There is a trend for an increase in placentome number from GD 40 to 60 which then remains unchanged after GD 60 (Ehrhardt and Bell 1995). In this study, we found a significantly lower number of placentomes in ewes with cyclopic fetuses versus ewes with normal fetuses. Additionally, using ultrasound imaging, we determined the maximum size of the placentomes to occur around GD 60 for ewes with monkey-faced fetuses and GD 75 for the remaining ewes (figure 2). Our results are similar to those in reported other studies wherein sheep placentas have been shown to display a period of maximal proliferative growth between GD 50-60 and an abrupt cessation of mass accumulation between GD 75-80 (Ehrhardt and Bell 1995). Interestingly, there was no difference in the size (diameter) of the placentomes in ewes with cyclopic fetuses as determined by ultrasound imaging or at necropsy.

In this study, 75 percent of the control ewes were found to be pregnant at GD 30 versus 87 percent of the treated ewes. However, there was not any embryonic, or fetal, loss (as determined by loss of fetus after a positive confirmation on GD 30) in control ewes versus a 25 percent loss in the treated ewes. A recent study using conditional knockout mice demonstrated that the Hedgehog signaling pathway plays an important role in implantation during pregnancy (Harman et al. 2011). In these mice, smoothened, the key Hedgehog pathway signal transducer, was conditionally deleted. Mice with one or two functional smoothened alleles had approximately 9 pups per litter whereas homozygous knockout mice had approximately 4 pups per litter. Interestingly, there was no difference in the average interval between litters or in the percentage of pups that were weaned. There was a significant reduction in the number of implantation sites in knockout mice compared to controls but no difference in the number of corpora lutea. Embryonic loss was not due to insufficient luteal function, as the knockout mice did not differ in serum progesterone concentrations during GD 4-13. The authors suggest that conditional reduction of Hedgehog pathway signaling due to lack of smoothened in the uterus leads to deferred implantation beyond the normal window of receptivity and that delayed implantation was associated with developmental delay in the embryos (Harman et al. 2011).

Normally, the ovine fetus exhibits an exponential growth pattern through GD 40-100. The fetus becomes the largest component of the gravid uterus near GD 100 and continues to gain size throughout the remainder of gestation (Ehrhardt and Bell 1995). In the study with conditional smoothened knockout mice, 20 percent of the fetuses in knockout mice were smaller than the wild type mice (Harman et al. 2011). The cyclopic fetuses assessed in this study were found to be significantly smaller than normal fetuses as determined by fetal weight, crown to rump length, abdominal and thoracic circumference, and fetal brain weight. Particularly striking was the large difference in the fetal brain weights. Several of the cyclopic fetuses had minimal brain tissue in the cranium with the presence of large amounts of fluid (figure 3). Consequently, with such lack of brain development, and likely function, the development of these fetuses would be severely compromised.

Another possible explanation for the delayed development in cyclopic fetuses could be due to defects in the pituitary in the fetuses and subsequently deficiencies in the production of hormones involved in growth and development. In early studies, many severely deformed cyclopic lambs did not have a pituitary gland (Binns et al. 1962). However, in this study, 55 percent of the cyclopic fetuses had a pituitary gland. Additionally, there was no difference in the size of the pituitary glands that were present in cyclopic fetuses versus those of normal fetuses. However, no histological or functional assessments of the pituitaries have been performed. Consequently, it is possible that the pituitary glands in the cyclopic fetuses were deficient in their ability to produce necessary hormones for normal development.

In summary, the results from this study confirmed that cyclopic fetuses are smaller than normal fetuses as well as less severely malformed

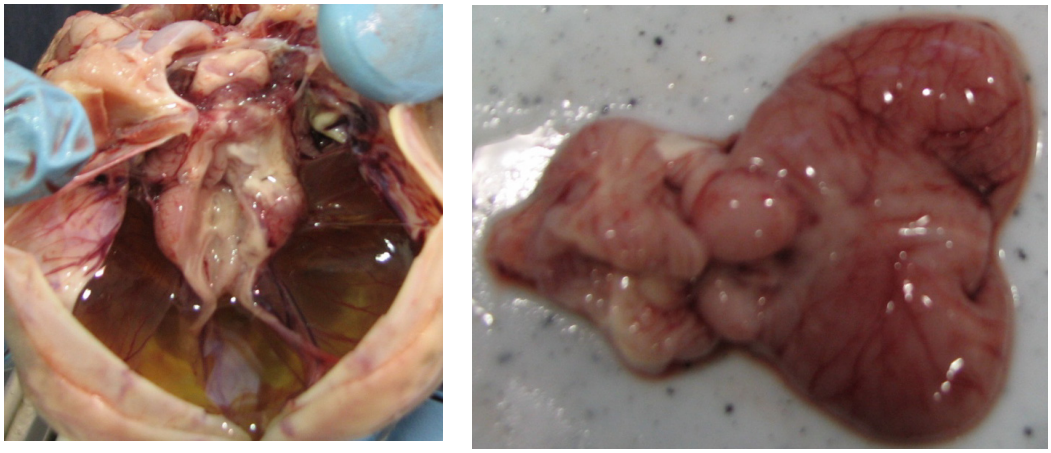

Figure 3. Fluid-filled brain cavity and brain from a cyclopic lamb.

fetuses. Additionally, we demonstrated that placental development in ewes with cyclopic fetuses is compromised. Due to the lack of brain and pituitary gland development in many of the cyclopic fetuses, it is quite likely that the lack of normal placental development in ewes with cyclopic fetuses is a result of insufficient contribution of the embryo during critical periods of placental development.

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