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Lysosomal storage disease induced by *Sida planicaulis* (Sin. *Sida carpinifolia*) (Malvaceae) in sheep in the state of Rio de Janeiro

[*Doença do armazenamento lisossomal induzida por* Sida planicaulis *(Sin.* Sida carpinifolia*) (Malvaceae) em ovinos no estado do Rio de Janeiro*]

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ABSTRACT

Ingestion of *S. planicaulis* (Sin. *S. carpinifolia*) causes lysosomal storage disease in sheep. The main toxic compound of this plant, swainsonine, inhibits the enzymatic activity of α-mannosidase I and II, resulting in lysosomal storage of glycoproteins. We describe a case of spontaneous poisoning by *S. planucaulis* in Rio de Janeiro state, Brazil. Physical examination of affected animals revealed proprioceptive deficit, motor incoordination, staggering movement, and head tremors. Histopathological evaluation showed severe swelling/cytoplasmic vacuolization in Purkinje neurons, with a foamy appearance and occasional karyolysis or karyopyknosis, and intense vacuolization of acinar cells of the pancreas and, less markedly, thyroid follicular cells. The positive lectin-histochemistry labeling for Con A, WGA and sWGA lectins characterized the disease as a glycoproteinosis. The ultrastructural evaluation revealed numerous vacuoles up to 2.5µm in diameter bounded by membranes up to 20nm thick in pancreatic acinar cells. The diagnosis of *S. planicaulis* toxicity was established based on epidemiological data, clinicopathological, lectino-histochemical, and ultrastructural findings. This is the second report of spontaneous poisoning of sheep by *S. planicaulis* in Brazil, but the first in the Southeastern Brazil.

Keywords: toxic plants, neurotoxicosis, Sida planicaulis, sheep

RESUMO

A ingestão de S. planicaulis *(Sin.* S. carpinifolia*) tem sido responsabilizada por doença do armazenamento lisossomal em ovinos. O principal composto tóxico dessa planta, a swainsonina, inibe atividade enzimática da α-manosidase I e II, que redunda no armazenamento de glicoproteínas no interior de lisossomos. Descreveu-se um caso de intoxicação espontânea por* S. planicaulis *no estado do Rio de Janeiro, Brasil. O exame físico dos animais afetados revelou déficit proprioceptivo, incoordenação motora, cambaleio à movimentação e tremores de intenção e na cabeça. A avaliação histopatológica evidenciou severa tumefação/vacuolização citoplasmática em neurônios de Purkinje, com aspecto espumoso e eventual cariólise ou cariopicnose e intensa vacuolização de células acinares do pâncreas e, menos marcadamente, foliculares da tireoide. O exame lectino-histoquímico positivo para as lectinas Con A, WGA e sWGA foi capaz de caracterizar a enfermidade como uma glicoproteinose. A avaliação ultraestrutural revelou numerosos vacúolos de até 2,5µm de diâmetro, delimitados por membranas de até 20nm de espessura em células acinares do pâncreas. O diagnóstico da intoxicação por* S. planicaulis *foi estabelecido com base nos dados epidemiológicos, nos achados clínico-patológicos e confirmado pelas avaliações lectino-histoquímica e ultraestrutural. Esta é segunda descrição da intoxicação natural por* S. planicaulis *em ovinos no Brasil e a primeira na região Sudeste do país.*

Palavras-chave: plantas tóxicas, neurotoxicose, Sida planicaulis, *ovinos*

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INTRODUCTION

Natural poisoning by *Sida planicaulis* (Sin. *Sida carpinifolia*) in Brazil has been described in ponies (Loretti *et al*., 2003), cattle (Furlan *et al*., 2008, 2009; Pedroso *et al*., 2010), sheep (Seitz *et al*., 2005a) and deer (Anjos *et al*., 2016). Toxicity occurs when *S. carpinifolia* predominates in the pasture (Driemeier *et al*., 2000; Colodel *et al*., 2002a) or when there is a marked lack of forage (Anjos *et al*., 2016). Animals can develop a special appetite for the plant after some time of grazing so that ingestion persists, even with the offer of other forages (Colodel *et al*., 2002b). The main toxic compound of this plant, swainsonine, inhibits the enzymatic activity of α-mannosidases I and II of the Golgi complex, which results in the storage of glycoproteins within lysosomes (Colodel *et al*., 2002b). This accumulation of substances promotes compression, deficiency in intracytoplasmic transport and degeneration in several tissues, especially the nervous system. The clinical signs observed in sheep are associated with ataxia, dysmetria, head and neck tremors, frequent falls, lateral decubitus, pedaling movements and difficulty in standing (Seitz *et al*., 2005a). Necropsy findings are nonspecific; lymphadenomegaly has been reported in sheep and goats (Driemeier *et al*., 2000; Colodel *et al*., 2002b). In sheep, the most frequent microscopic findings consist of marked distension and multiple cytoplasmic vacuolizations in Purkinje neurons of the cerebral cortex, thalamus, midbrain, and spinal cord ventral horn. Axonal spheroids are also seen in the granular layer of the cerebellum and other areas of the brain and spinal cord. There is cytoplasmic vacuolization in pancreatic acinar cells, renal tubule epithelial cells, thyroid follicular cells, hepatocytes, and macrophages in lymphoid organs (Seitz *et al*., 2005a). Lesions of the same nature have also been observed in goats (Driemeier *et al*., 2000) and cattle (Furlan *et al*., 2009). Histological findings compatible with intoxication were also found in fetuses of goats and cattle intoxicated with *S. carpinifolia* (Pedroso *et al*., 2012). The ultrastructural findings are characteristic of a storage disease, with finely granular vacuoles delimited by thin membranes in the cytoplasm of neurons (mainly Purkinje) and small neurons of the granular layer of the cerebellar cortex, pancreatic acinar cells, thyroid follicular cells and hepatocytes (Seitz *et al*. 2005a). Sometimes, cattle present vacuolization of thalamic neurons (Pedroso *et al*., 2010). The present work aims to describe the histological, lectin-histochemical and ultrastructural aspects of natural poisoning by *Sida planicaulis* in sheep. Natural toxicity by *S. planicaulis* in sheep has not yet been described in the Southeast region of Brazil.

MATERIAL AND METHODS

Natural poisoning occurred in a sheep breeding farm in Nova Iguaçu, RJ (22°50'41" S, 43°38'39" W) from September of 2014 to December of 2017. The Anatomic Pathology Section(SAP-UFRRJ) team was asked to investigate problems arising from the ingestion of a plant until then referred to as "broom". During visits to the property, we identified sick animals and performed clinical examinations and necropsies. In addition, we evaluated the pasture and harvest conditions for botanical identification. Samples of the suspected plant were collected, pressed on absorbent paper, changed daily until the specimen was completely dry (14 days) and sent to the Herbarium of the Department of Botany at the Universidade Federal Rural do Rio de Janeiro for botanical identification and deposited as voucher number RBR 42728.

Sections of the nervous system, pancreas, thyroid, lymph nodes, liver, gastrointestinal tract, heart, spleen, bladder, and placenta were fixed in 10% formalin, dehydrated in alcohol, diaphanized in xylol and embedded in paraffin blocks for microtome cutting. Three μm sections of all tissues were stained by HE. Histological sections of the cerebellum were also stained with Luxol Fast Blue (LFB) and Cresil Violet (CV).

For the lectin-histochemical evaluation, sections of the nervous system, pancreas, liver and thyroid obtained on silanized slides, were dewaxed, hydrated in alcohol and incubated in 3% hydrogen peroxide solutions to block endogenous peroxidases; then, sections were washed (2 min.) with PBS and immersed in citrate buffer (pH 6.0) for antigen recovery for 15 minutes at 98 ºC in a water bath, with subsequent cooling at room temperature for 15 minutes. To block nonspecific reactions, 5% skimmed milk (Molico® - Indústria Brasileira) was used. The sections were incubated for 12 to 14 hours overnight with lectins (Lectin Kit

Biotinylated BK 1000 and 2000 - Vector Laboratories, Burlingame, California, 94010, USA) at a dilution of 5µg/mL in PBS, except the Con A lectins, which was diluted in the proportion of 0.5µg/ml and the RCA lectin diluted to 1µg/ml. Next, the sections were exposed to the Vectastain Elite ABC Development Kit (Vector Laboratories Inc.) for 30 minutes and Diaminobenzidine (DAB) chromogen. The sections were counterstained with Harris hematoxylin and evaluated under an optical microscope through semi-quantitative analysis with graduations according to the intensity of marking found, being: (-) marking absent, (+) discrete, (++) moderate and (+++) intense. The sections were evaluated in a Zeiss Axio Scope A1 microscope and the photomicrographs were captured with a 5-megapixel Zeiss Axiocam IC5 camera, using the Zen Blue Lite software.

Sections of the central nervous system embedded in paraffin were sent to the Department of Veterinary Pathology of the Federal University of Rio Grande do Sul (UFRGS) for immunohistochemical examination of Sheep Spongiform Encephalopathy (Scrapie) by standard procedure of the lab (Leal *et al*., 2012).

For ultrastructural transmission analysis, 1-2mm^3 fragments of the pancreas after fixed in 3% buffered glutaraldehyde in 0.166 M sodium cacodylate solution (Electron Microscopy Sciences, Hatfield, Pennsylvania), and refixed in osmium tetroxide to 1% was sent to the Diagnostic Laboratory at the University of Veterinary Medicine of Minnesota, USA, for routine processing. The dimensions of cell structures were analyzed using the iTEM software (Olympus SIS, Munster, Germany).

RESULTS

Through the period of the outbreak, we visited the property in four occasions. We were informed that the property had been recently acquired, with Santa Inês sheep breed and crossbred farming. Data about the animals was scarce regarding the origin, accurate number, and time of acquisition. The herd reached a number close to 50 sheep, kept in the same lot. According to the owner, un uncertain number of sheep showed tremors, difficulty in walking, falling, inability to stand, progressive weight loss, weakness, and retarded growth. Pasture inspection revealed large amounts of *Sida planicaulis*, diffusely distributed, with signs of grazing. The dry period combined with difficulties in establishing good irrigation of pastures and grassland generated a low forage supply. For a long period, the animals had *S. planicaulis* as their main food source, especially the younger animals that competed for forage with the adults. One year after the first diagnosis, the pasture still had insufficient irrigation, management-related problems were still frequent (pastures invaded by *S. planicaulis* and forage shortage) and some animals still presented clinical signs compatible with intoxication by *S. planicaulis*. After supply of alternative forage, clinical signs were considered milder in affected sheep, and three years after start of the outbreak, no affected sheep were identified, although there was still marked invasion of pastures by *S. planicaulis*. The absence of effective zootechnical bookkeeping and the refusal of the owner to provide more accurate information made it impossible to establish precise rates of morbidity, mortality, and lethality of the disease in the herd.

Sheep 1 (female, 5 years-old) evaluated during the first visit had severe lethargy, cachectic, lying down, poorly responsive to external stimuli and swollen carpal joints. During the second visit, 9 other animals demonstrated staggering walking, ataxia and low body score. Some sheep (adults and lambs) had mild head tremors, which were regular at rest and intensified during feeding and handling. When moved, the most affected sheep had gait disturbances ranging from mild to severe dysmetria with hypermetria and falls. In sheep with gait disturbances, the tremors were more pronounced. Sheep apparently healthy, when stressed by any situation, such as movement, occlusion of vision or after application of the "Head Raising Test" (HRT - Head Raising Test), started the tremors of intention. After the application of HRT, some sheep became "dizzy", fell to the ground, and had difficulties in getting up for a few seconds. Neurological examination of the four most affected animals, three females (sheep 2, 3 and 4 with six, four and five years old, respectively) and one male (sheep 5, 6 months old) revealed a proprioceptive deficit characterized by abduction of thoracic and pelvic limbs or changes in the position of the paws, motor incoordination and

intention tremors. The owner chose to euthanize sheep 1, 2, 3, 4 and 5. At the necropsy of sheep 1, an increase in volume in the radiocarpal joints, with thickening of the periarticular tissue, lymphadenomegaly (mesenteric and pre-scapular) was observed and mild parasitosis by *Haemonchus contortus*. Sheep 2, 3, 4 and 5 showed no noteworthy changes at necropsy.

The most significant histopathological findings in the five necropsied sheep occurred in the central nervous system; there was vacuolization/swelling of neurons in the Purkinje layer of the cerebellum (5/5), in the pyramidal layer of the cortex (1/5), in the inner capsule (1/5) and nuclei of the base (1/5). Astrocytic proliferation with bi- or trinucleated astrocytes, in the process of amitosis, with margined chromatin and indented nuclei were observed in the telencephalic cortex (3/5), internal capsule (2/5), thalamus (2/5), midbrain (4/5), bulb and pons (2/5), cerebellum (2/5) and ventral horns of the spinal cord (3/5). Usually, this proliferation became more intense around necrotic neurons (satellite) or in an advanced degenerative process (neurons in chromatolysis or with pyknotic nuclei and with strongly eosinophilic cytoplasm, sometimes swollen). There was also axonal degeneration with spheroid formation in the thalamus (1/5), midbrain (2/5), brainstem (1/5) and telencephalic cortex (1/5). LFB staining detected mild demyelination in portions of the cerebellum and CV staining demonstrated severe loss of Nissl substance in Purkinje neurons. There was also multifocal vacuolization of pancreatic acinar cells (4/5) and thyroid follicular cells (4/5), as well as mild and moderate swelling/microvacuolization of the renal tubular epithelium (2/5). The histopathological findings observed in the fetus of Sheep 4 and placenta were restricted to the moderate vacuolization of the epithelial cells of the renal tubules and cytoplasmic vacuolization of the chorionic epithelium.

Histological sections of the cerebellum submitted to lectin-histochemical evaluation revealed, for the Con A lectin, variable reactivity between mild and marked in the cytoplasmic vacuoles of Purkinje neurons and moderate in the cytoplasm of Golgi neurons. Regarding the WGA lectin, there was strong reactivity in Purkinje neurons cytoplasmic vacuoles and mild to moderate Golgi neurons. There was strong reactivity to the sWGA lectin in the cytoplasm of neurons of the nuclei of the base of the cerebellum (bulb and pons) and weak immunostaining in the cytoplasm of Purkinje neurons. The PSA lectin showed moderate reactivity in the cytoplasm of Purkinje neurons. Other lectins did not show specific markings on vacuoles. Pancreatic acinar cells showed strong reactivity for intracytoplasmic vacuoles in the lectin-histochemical evaluation for PHA-L, PHA-E and SBA, moderate reactivity for Con A, WGA, DBA and LCA, and weak for sWGA. Thyroid follicular cells did not show reactivity to the tested lectins. Sections of placentomes were negative to all the lectins tested.

In the immunohistochemical examination, there was no reactivity to the anti-prion antibody of Sheep Spongiform Encephalopathy (Scrapie) in the evaluated cerebellum sections.

The semi-thin sections of the pancreas revealed severe intracytoplasmic vacuolization in acinar cells, which under transmission electron microscopy were characterized as vacuoles up to 2.5μm in diameter, mostly empty, but some had amorphous, reticular substance and osmiophilic granules. These vacuoles were delineated by a thin single-layer membrane measuring up to 20nm in thickness, characteristic of lysosomes.

DISCUSSION

Diagnosis of poisoning by *Sida planicaulis* Cav. (S. carpinifolia) was established through epidemiological data, associated with histopathological findings, and confirmed by lectin-histochemical and ultrastructural evaluations. In the observed outbreak, the decrease in the supply of forage, combined with the severe invasion of *S. planicaulis* in pastures, induced the consumption of the plant in large quantities and, consequently, caused the disease, especially in weaker animals in the herd hierarchy, as previously observed in captive deer (Anjos *et al*., 2016). Mistakes in pasture management and overcrowding are reported as important factors in the outbreak of outbreaks involving *S. planicaulis* (Colodel *et al*., 2002a; Furlan *et al*., 2009; Pedroso *et al*., 2010; Anjos *et al*., 2016); some animals develop a taste for the plant and subsequently ingest it even with the availability of another forage (Furlan *et al*., 2009; Tokarnia *et al*., 2012), as already seen in

poisoning by other plants that contain swainsonine (Barbosa *et al*., 2006; Armién *et al*., 2007; Dantas *et al*., 2007).

The clinical signs were similar to those already observed in sheep naturally intoxicated by *S. planicaulis* (Seitz *et al*., 2005a), and in cattle (Furlan *et al*., 2008, 2009; Pedroso *et al*., 2012), goats (Driemeier *et al*., 2000; Colodel *et al*., 2002a), horses (Loretti *et al*., 2003; Bassuino *et al*., 2017) and naturally intoxicated deer (Anjos *et al*., 2016).

Optical microscopy of the nervous system revealed severe intracytoplasmic swelling/vacuolation in neurons at different areas in the central nervous system, especially in the Purkinje layer of the cerebellum, whose malfunction is responsible for the cerebellar syndrome. (Oliveira Jr *et al*., 2013). There are also degenerative-necrotic changes in cerebellar, bulbar, and pontine nuclei that participate in the pathogenesis of the observed signs. As in other studies (Colodel *et al*., 2002b; Seitz *et al*., 2005a; Furlan *et al*., 2008, 2009), there was severe vacuolization in pancreatic acinar cells and thyroid follicular cells, however without clinical changes associated with dysfunction of these organs. The histopathological analysis of the fetus revealed swelling/vacuolation in the epithelial cells of the renal tubules, in the thyroid follicular cells and the placentomes, as observed in cows naturally poisoned by *Sida carpinifolia* (Reis *et al*., 2019), however, the lectin-histochemical technique was not able to identify oligosaccharides in these tissues, a fact already mentioned in placentas and fetuses of sheep intoxicated by *Ipomoea carnea* subsp. *fistulosa* (Armién *et al*., 2011). It cannot be ruled out that other substances can be stored, due to the intrinsic nature of the metabolism of these cells.

Reactivity to Con A, WGA and sWGA in affected neurons and pancreatic acinar cells, indicated the presence of α-glucose, α-mannose and β-n-acetylglycosamine residues. The labeling pattern was similar to that described in congenital mannosidosis and other storage disease-causing plants (Alroy *et al*., 1985, Driemeier *et al*., 2000; Seitz *et al*., 2005b; Armién *et al*., 2007; Pedroso *et al*., 2012; Bassuino *et al*., 2017). Additional positive labeling of pancreatic acinar cells for the lectins PSA, PHA-E, PHA-L, DBA, LCA and SBA indicates storage of α-glucose, α-mannose, α-N-acetylgalactosamine and complex sugars.

On ultramicroscopic examination, vacuolization within pancreatic acinar cells, covered by simple layer membranes up to 20nm in width, are characteristic of lysosomes, confirming the nature of the vacuoles observed by optical microscopy, similarly to that found in goats, sheep and cattle intoxicated by *S. planicaulis* (Driemeier *et al*., 2000; Seitz *et al*., 2005a; Furlan *et al*., 2008, 2009) and in goats intoxicated by *Ipomoea carnea* ssp. *fistulosa* (Armién *et al*., 2007).

The main differential diagnoses to be considered are diseases that can occur with tremors and/or ataxia in sheep. Copper-deficient enzootic ataxia can be ruled out, as it affects lambs up to four months of age (Tokarnia *et al*., 2010) and causes demyelination in the spinal cord and splenic hemosiderosis. The clinical and pathological findings of intoxications by other plants that cause glycoproteinosis are identical to those observed in this outbreak, however the massive invasion of numerous specimens of *S. planicaulis* in pasture, with signs of grazing, coupled with the absence of *Ipomoea carnea* ssp. *fistulosa* in that property, allows the diagnosis of poisoning by *S. planicaulis*. The other plants in this group, *Turbina cordata*, *I. sericophylla*, *I. riedelli*, *I. verbascoideae*, found in the northeast region (Oliveira Jr *et al*., 2013), were not present in the pasture. Intoxication by *Ipomoea asarifolia* causes neurological symptoms in sheep, in part, similar, with tremors, hypermetry, ataxia, staggering gait and falls (Araujo *et al*., 2008), however in intoxication by *I. asarifolia* there is no storage of oligosaccharides and *I .asarifolia* was also not found in the pasture. Mycotoxicosis by *Claviceps paspali* only occurs in *Paspalum* spp pastures (Riet-Correa *et al*., 2013) and does not present the characteristic lesions of storage diseases. The diagnostic possibilities of mycotoxicosis caused by *Stenocarpella maydis* and *Aspergillus clavatus* were ruled out since there was no history of administration of corn or barley to the sheep in question and the histopathology did not show demyelination and spongiosis, common in diplodiosis, as well as were not observed chromatolytic neurons typical of *A. clavatus* toxicosis (Riet-Correa *et al*., 2013; Ribeiro, 2016). Ovine spongiform encephalopathy is a

disease that causes vacuolization and microvacuolization in the nervous system, however, these changes occur both in neurons and in the neuropil (Miller and Zachary, 2017), which was not verified in the present case. In addition, there was no reactivity for the anti-prion antibody in the immunohistochemical examination and this disease was ruled out. The swollen joint in sheep 1 is probably associated with mycoplasmosis, endemic in the Region.

CONCLUSION

The epidemiological and clinicopathological data associated with the results of the lectin-histochemical and ultramicroscopic exams confirm *Sida planicaulis* (*S. carpinifolia*) as the cause of the outbreak of poisoning in sheep observed in the State of Rio de Janeiro. This intoxication, as in Rio Grande do Sul, should be considered as a differential diagnosis of diseases of the central nervous system in sheep in the Southeast Region, especially in conditions of marked lack of forage even outside the dry season when the plant predominates in the pasture.

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