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Experimental intoxication of guinea pigs with *Ipomoea carnea*: Behavioural and neuropathological alterations

Luciana A. Cholich [a,*], Mercedes Márquez [b], Martí Pumarola i Batlle [b], Eduardo J. Gimeno [c], Gladys P. Teibler [a], Elvio E. Rios [a], Ofelia C. Acosta [a]

[a] UNNE, Faculty of Veterinary Sciences, National University of Northeast, Corrientes, Argentina
[b] UAB, Facultat de Veterinària, Universitat Autònoma de Barcelona, Spain
[c] UNLP, Faculty of Veterinary Sciences, National University of La Plata, Argentina

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ABSTRACT

Ipomoea carnea is a toxic plant that affects goats, with symptoms being characterised by nervous disorders and death. Swainsonine and calystegines are the principal toxic components isolated from *I. carnea*, which also yields lysergic acid derivatives. The aim of this study was to improve the clinical characterisation of experimental intoxication by *I. carnea* in guinea pigs through the evaluation of behavioural changes and to perform a thorough histopathological analysis of the affected CNS. Leaves of *I. carnea* were administered to guinea pigs. Open-field gait analysis and monoamine levels were measured. The poisoned animals exhibited increased vocalisation, lethargy, and a reduction in the locomotion frequency after the fourth week of intoxication, as demonstrated in the open-field test. Significant differences were observed in hind-limb gait width by the last week of intoxication. After 65 days, the guinea pigs were euthanised, necropsied, and examined using light and electron microscopy. At the end of the experiment, plasma serotonin decreased. In contrast, dopamine decreased, and noradrenaline increased in urine. Brain sections were evaluated with conventional histological methods and immunohistochemistry (IHC), as well as by transmission electron microscopy (TEM). Vacuoles were observed throughout the brain, but they were particularly prominent in the brainstem. In addition, there were PAS-negative regions, and the Nissl substance was dispersed or absent, which was confirmed with the Kluver-Barreda stain. Moderate microgliosis was observed by immunohistochemistry. In the medulla oblongata, numerous ubiquitin-positive spheroids together with neuronal degeneration were observed in the nucleus gracilis/cuneatus. Furthermore, vacuoles were observed in astrocytes, oligodendrocytes, and endothelial cells by TEM. Our results showed that the behavioural effects may have been caused by alterations in the brain in conjunction with changes in monoamine levels. This research confirms the utility of this model for studying the pathogenesis of plant-induced lysosomal storage diseases.

1. Introduction

Ipomoea carnea subsp. *fistulosa* (Convolvulaceae) is a toxic plant found throughout the northeastern region of Argentina and in other tropical and subtropical countries (Austin and Huaman, 1996). Poisoning occurs when various animal species, such as goats, sheep, and cattle, eat this

* Corresponding author. Tel./fax: +54 3783 425753x146.
E-mail addresses: lcholich@vet.unne.edu.ar, lucianacholich@hotmail.com (L.A. Cholich).

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plant, especially in drought periods, when it is one of the few plants that stay green (Riet-Correa and Mendez, 2000).

Prolonged ingestion of this and similar plants induces neurodegeneration characterised by neuropathy and other clinical manifestations. The animals exhibit a variety of clinical signs, such as addiction to consumption of the plant, depression, and loss of body weight, and neurological deficits, such as abnormalities of gait, difficulty standing, abnormal posture, symmetrical ataxia, and paraparesis (Barbosa et al., 2006; de Balogh et al., 1999; Dorling et al., 1980; Driemeier et al., 2000; James et al., 1970; James and Panter, 1989; Molyneux et al., 1995; Ríos et al., 2012; Rodriguez Armesto et al., 2004; Stegelmeier et al., 1999).

These toxic effects are attributed to the polyhydroxylated alkaloids swainsonine and the calystegines A_3, B_1, B_2, B_3, and C_1. Swainsonine is a powerful inhibitor of lysosomal α-mannosidase and Golgi mannosidase II. Calystegines inhibit the lysosomal α-galactosidase and β-glycosidase (Colodel et al., 2002; de Balogh et al., 1999; Haraguchi et al., 2003; Hueza et al., 2005; Molyneux et al., 1995). The inhibition of lysosomal α-mannosidase leads to the intralysosomal accumulation of incompletely processed oligosaccharides. These enzymes are essential for the proper functioning of all animal cells, and their inhibition results in a clinical, biochemical, and morphological presentation similar to inherited-α-mannosidosis (Dorling et al., 1978; James et al., 2004). These alkaloids were isolated from *I. carnea* found in Corrientes, Argentina with HPLC-MS (Cholich et al., 2009).

Furthermore, several authors reported the presence of lysergic acid derivatives in *Ipomoea* (Daló and Moussatché, 1978; Sandoval et al., 2010).

Congenital α-mannosidosis has been reported in humans, cattle, cats, mice, and guinea pig, and this disease is characterised in all species by progressive neurological deterioration and premature death (Auclair and Hopwood, 2007; Crawley and Walkley, 2007; Robinson et al., 2008). Plant-induced α-mannosidosis has been experimentally studied in goats, cattle, and sheep, as well as in rats and mice (Van Kampen and James, 1969; Riet-Correa and Mendez, 2000; Hueza et al., 2005; Armién et al., 2007; Stegelmeier et al., 2008). Nevertheless, it was found that small rodents are poor models for neuronal storage diseases. Vacuolation in various tissues but not central nervous tissue were obtained with the aqueous fraction of *I. carnea* and, to a lesser degree, with swainsonine alone, but no effect was found with the individual calystegines (Hueza et al., 2005). Stegelmeier et al. (2008) reproduced neuronal storage defects in mice but with high doses of pure swainsonine administered subcutaneously by osmotic minipumps.

Recently, we proposed that *I. carnea*-induced toxicosis in guinea pig could be a useful model for studying the pathogenesis of plant-induced storage diseases (intoxications with the genera *Ipomoea, Swainsona, Astragalus, Oxytropis, Sida*, and others) and for plants with similar effects that are still awaiting characterisation (Cholich et al., 2009).

The aim of this study was to improve the clinical characterisation of the disease in intoxicated guinea pigs, especially by the evaluation of behavioural changes and assaying monoamine levels, and to perform a thorough histopathological study of the guinea pig CNS by light microscopy and transmission electron microscopy (TEM).

2. Materials and methods

2.1. Preparation of plant material

The plant was identified as *I. carnea* subsp. *fistulosa* from the Convolvulaceae family, known in Northern Argentina with the common Guarani names of "mandiyura'" or "aguapeí". Polyhydroxy alkaloids were detected as chemical constituents of these plants, including swainsonine, calystegine B1, calystegine B2, calystegine C1, and trace amounts of calystegines A3 and B3. The swainsonine concentration was 0.02%, whereas the mean levels of calystegine were 0.05% (Cholich et al., 2009).

I. carnea leaves were dried al 37 °C for 72 h and milled. The leaves were mixed homogeneously with commercial guinea pig pellets, previously hydrated with water for making "small balls". A 50:50 (vol/vol) mixture of pellets and dry, milled leaf matter was used in this study.

2.2. Clinical study

Eight 4-week-old male Hartley guinea pigs (253 ± 22 g) were supplied by the Bioterio of the Faculty of Veterinary Sciences, UNNE. The animals were housed individually in a temperature-controlled room (23 ± 2 °C) with relative humidity between 35 and 65%. Lights in the animal room were on from 6 AM to 6 PM. The animals were divided at random into two groups: experimental group ($n = 4$), each received 5 "small balls" (10 g each) per day (total swainsonine, 1.25 mg). In the control group ($n = 4$), each animal received 50 g commercial pellets per day.

Both groups received both fresh alfalfa (*Medicago sativa*) and water *ad-libitum*. Through the consumption period, the animals were weighed weekly, and the daily intake of small balls and commercial pellets was calculated based on subtraction of food on the next day.

The present study was approved by the Ethics at Biosafety Committee of Facultad de Ciencias Veterinarias, Universidad Nacional del Nordeste, Argentina.

2.3. Neurological examinations

2.3.1. Open-field studies

This type of study is commonly used to assess locomotion and exploration in laboratory animals. Each animal was individually placed in the centre of a box (120 × 120 × 50 cm) in which the floor was divided into 16 units of 30 cm^2 each, and the following parameters were measured over a period of 5 min: locomotion frequency (number of floor units entered with all feet), rearing frequency (number of times the animal stood on its hind legs), immobility time (total number of seconds with no movement) and defecation (number of faecal pellets). Hand-operated counters and stopwatches were employed to score these behaviours. To minimise possible influences of circadian changes on open-field behaviours, control and experimental animals were alternated. The device was washed with a 5% alcohol/water solution before the

animals were placed on it, to obviate possible biasing effects due to odour clues left by previous guinea pigs (Schwarz et al., 2003).

2.3.2. Gait analysis

Gait analysis is a method for assessing signs of ataxia or motor incoordination through the hind limb gait of animals. Gait was analysed using a long box (approximately 1 m long by 0.3 m wide), high at the sides and open at the top, with a dark enclosure at one end, containing food (alfalfa) as an incentive. Paper for recording gait patterns was placed on the floor of the box. The hind paws of the guinea pigs were dipped in non-toxic food colouring, and the animals were later placed at the open end of the box such that they would walk along the paper to the dark enclosure. Animals were tested until two clean gait patterns (i.e., animal walking all the way along the paper) were obtained for each animal at each time. Gait length and width were measured manually from the footprints collected (Robinson et al., 2008).

2.4. Determination of monoamine neurotransmitters

Venous blood and urine, obtained by bladder puncture, were collected from control and experimental guinea pigs. Immediately before they were euthanised, guinea pigs were anesthetised by an intramuscular injection of ketamine hydrochloride and xylazine hydrochloride. The quantification of urinary noradrenaline (NA) and dopamine (DA) and plasma serotonin (5-HT) was performed with methods based on high performance liquid chromatography, with electrochemical detection (Hou et al., 2002).

2.5. Post-mortem study

After 65 days of intoxication, the animals were euthanised as a consequence of the severity of the symptomatology. Brains were removed and fixed in 10% formalin for 24 h. Then, they were cut into five transverse sections at the following levels: anterior to the optic chiasm, caudal to the pituitary gland, midbrain, cerebellum-pons and medulla oblongata. Specimens were embedded in paraffin, sectioned at 5-μm thicknesses, and stained with haematoxylin and eosin (HE), periodic acid-Schiff (PAS) for carbohydrates and Kluver-Barrera (KB), to detect lysosomal vacuolation in brain regions and to stain myelin.

Immunohistochemistry was performed using paraffin-embedded 5-μm sections. Antigen retrieval was conducted by pretreatment of the sections with citrate buffer, pH 6, at 96–98 °C for 20 min. Primary antibodies and working dilutions were as follows: polyclonal anti-ubiquitin 1:2000 (Z0458, DakoCytomation, Glostrup, Denmark), polyclonal anti-glial fibrillary acidic protein (GFAP) 1:1500 (Z0334, DakoCytomation), goat polyclonal anti-brain microglia 1:600 (anti Iba-1, ab5076 Abcam, Cambridge, MA, USA), monoclonal anticalbindin-D-28K 1:500 (clone CB 955, Sigma–Aldrich, St. Louis, MO, USA) and monoclonal anti-parvalbumin 1:50 (clone PARV-19, Sigma–Aldrich). The anti–mouse (K4007, DakoCytomation) and anti-rabbit (K011, DakoCytomation) EnVision kits, and the polyclonal rabbit anti-goat serum (E0466, DakoCytomation) + ABS system, were used to label the primary antibodies. In all cases, the diaminobenzidine-based detection kit from DakoCytomation was used. Omission of the primary antibody was used as a negative control, and the positive controls included CNS from control guinea pigs, to which the appropriate antisera were added.

2.6. Transmission electron microscopy

Immediately after sacrifice, the brain was fixed by immersion in cacodylate buffered 2% glutaraldehyde until sectioning it into five transverse sections, as described before. Then, they were post-fixed in osmium tetroxide and embedded in epoxy resin (EPON™ Momentive Specialty Chemicals Inc., Columbus, OH, USA). Semi-thin sections (1 μm thick) were stained with 1% toluidine blue in 1% borax. Ultra-thin sections (60–80 nm) of selected areas were stained with 2% uranyl acetate and lead citrate, and examined in a transmission electron microscope (JEOL EM 1200EX II, Tokyo, Japan).

2.7. Statistical analysis

Values are expressed as means ± standard deviation (SD). Data were analysed statistically by one-way analysis of variance (ANOVA). When the values were significantly different ($p < 0.05$), the differences between pairs of means were analysed by the *t*-test. Statistical analysis was performed using Infostat software.

3. Results

3.1. Clinical signs

Some clinical signs observed in our poisoned guinea pigs have already been previously described, such as hirsutism, emaciation and body weight loss (Cholich et al., 2009). In this study, there was a significant difference in food consumption but no significant difference in water consumption between the experimental groups (Table 1). Furthermore, from the second week after the beginning of the experiment, the intoxicated animals vocalised more when people entered the animals' room. They stopped when they were provided with "small balls" of powdered leaves and the fresh alfalfa. Starting in the fourth week, the vocalisation persisted until these animals refused the alfalfa and eagerly sought the small balls. When the alfalfa was put

Table 1
Total water and food consumption per day, from animals treated with small balls, during the 65 days.

	Day	Untreated animals ($n = 4$)	Treated animals ($n = 4$)
Water consumption (ml)	0–20	41.08 ± 0.64	40.69 ± 8.68
	20–40	46.39 ± 5.56	41.37 ± 5.70
	40–65	45.49 ± 3.52	42.77 ± 4.58
Food onsumption (g)	0–20	29.22 ± 5.17	15.20 ± 5.51[a]
	20–40	32.14 ± 1.80	30.84 ± 2.39
	40–65	30.72 ± 2.17	31.83 ± 1.29

[a] Data are presented as means ± S.E.M.*$P < 0.05$ compared to untreated animals.

on one side and the balls on the other side, even the animals with impaired mobility went toward the balls, demonstrating preference behaviour in poisoned guinea pigs. They increased vocalisation if the "balls" were taken away from them. After the fourth week, neurologic deterioration was progressive and showed increased severity. The control animals showed no clinical signs during the experiment.

3.2. Neurobehavioral findings

3.2.1. Open-field studies

The locomotion frequency, as shown by the *t*-test, was reduced from the fourth week onward in the poisoned animals ($p < 0.0142$) as compared to the controls. An increase in immobility time in poisoned animals compared to controls was also observed ($p < 0.0299$). No difference between groups was observed in rearing frequency or defecation (Fig. 1 A, B).

3.2.2. Gait analysis

Treated guinea pigs moved less smoothly than normal animals and showed poorer coordination. In the last week, the intoxicated animals showed a decreasing trend (in cm) in stride length (9.76 ± 1.47; $P \geq 0.068$), compared to normal animals (12.45 ± 1.93) and significant increases in stride width (6.15 ± 0.78; $P \leq 0.0431$) when compared to control animals (4.99 ± 0.46).

3.3. Quantitative monoamine determinations

The levels of neurotransmitters were determined on day 65, the end of the experiment. The plasma serotonin concentration was observed to decrease, whereas noradrenaline increased and dopamine decreased in urine (Table 2).

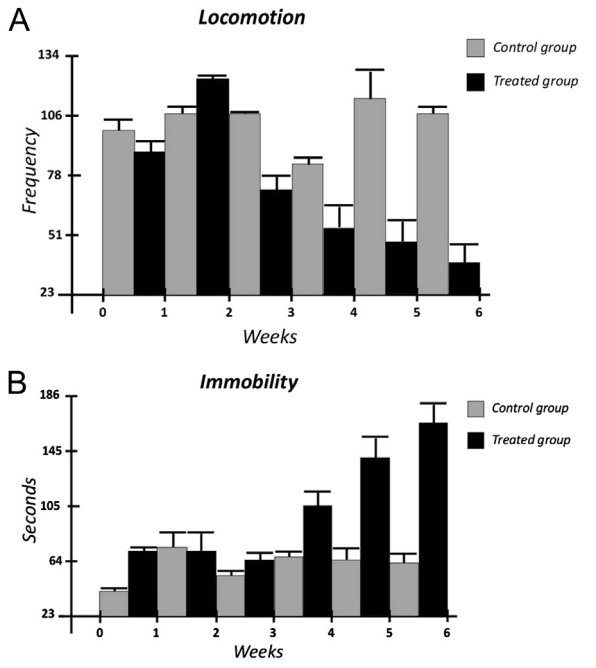

Fig. 1. Behavioral effects of *I. carnea* in guinea pigs exposed to "small balls" and control animals during 65 days. (A) Effects on locomotion frequency. (B) Effects on immobility. Data are presented as means ± S.E.M; $n = 4$ per group. $p \geq 0{,}05$.

3.4. Microscopic lesions

At necropsy, the gross findings in poisoned guinea pigs included muscle atrophy, loss of fatty tissue and pale mucous membranes. No macroscopic lesions were observed in the brain. Examination of HE-stained sections revealed widespread changes throughout the brain, but there were particularly prominent changes in the brainstem. Generally, the neurons had a distended perikaryon containing numerous large, pale and apparently empty vacuoles; however, Purkinje cells, septal nuclei and thalamic neurons exhibited few small vacuoles (Table 3). There were also clearly defined vacuoles in ependymal cells of the choroid plexus (Fig. 2 A, B). No vacuoles were observed inside glial cells. The content of vacuoles was negative for both PAS and KB stains (Fig. 2 C, D).

In certain cases, the nuclei of affected cells were pyknotic and eccentric with no apparent nucleolus and dispersed or absent Nissl substance (chromatolysis), as corroborated by KB staining. In the medulla oblongata of affected animals, numerous spheroids, together with neuronal degeneration, were observed in the gracile nucleus, and, to a lesser extent, in the cuneate nucleus. Both degenerate neurons and axonal spheroids were strongly positive for ubiquitin immunostaining (Fig. 3 A, B) but negative for calbindin and parvalbumin. No differences of myelin intensity with Kluver-Barreda luxol stain (KB) were observed between groups. Immunohistochemical studies showed moderate microgliosis (Iba-1 labelled cells) (Fig. 3 C, D) but no astrogliosis (GFAP).

3.5. Ultrastructural findings

The ultrastructure of the CNS showed different degrees of vacuolisation in the perikarya of neurons in poisoned animals. Most vacuoles were optically empty, but some of them were filled with membrane fragments, vesicles, reticular or dense granules, amorphous substances, opaque globules or osmiophilic material. Similar vacuoles were also found in astrocytes, oligodendrocytes and endothelial cells (Fig. 4 A, B).

4. Discussion

Our results indicate that *I. carnea* ingestion in guinea pigs induced neurological toxicity with physical and behavioural impairment. Specific neuropathological lesions

Table 2
Levels of plasma serotonin (5-HT) and urine dopamine (DA) and noradrenaline (NA) in guinea ping poisoned with *I. carnea*.

	Monoamine transmitters		
	DA (ug/24 h)	Na (ug/24 h)	5-HT (ng/ml)
Treated	12.03 ± 1.10^{a}	18.63 ± 1.46^{b}	28.17 ± 3.53^{c}
Control	41.27 ± 4.32	1.60 ± 0.52	144.20 ± 3.54

[a, b c] values were statistical significance in treated group (a $p < 0{,}0003$, b $p < 0{,}0001$, c $p < 0{,}0001$), when compared to values of controls.

Table 3
Neuronal cytoplasmic vacuolation in brain regions of poisoned guinea pigs in haematoxylin and eosin-stained paraffin sections.

Region	Cell type	Neuronal cytoplasmic vacuolation
	Septal nuclei	+
	Thalamic neurons	+
Cerebellum	Fastigial nuclei	++
	Purkinje cell	+/++
Midbrain	Red nucleus	++
	Pretectal nuclei	++
	Inferior colliculus	+
	Oculomotor Nuclei	++/+++
	Cerebral crus	+
Pons	Vestibular nuclei	++/+++
	Cochlear nuclei	+
Medulla Oblongata	Solitary nucleus	+++/++++
	Hypoglossal Nucleus	+++
	Reticular formation	+++/++++
	Trigeminal nerve nuclei	+++
	Cuneate Nucleus	++/+++
	Nucleus ambiguous	++/+++
	Olivary nucleus	−/+
	Gracile nucleus	++
	Facial nucleus	+
	Choroid Plexus	++

−/+, low numbers of vacuoles in rare numbers of cells; +; low numbers of vacuoles or low numbers of cells moderately affected; ++, moderate numbers of vacuoles in many cells; +++/++++, many vacuoles distending cytoplasm in majority of cells.

and changes in concentrations of neurotransmitters were observed: serotonin decreased in plasma, whereas dopamine decreased and noradrenaline increased in urine.

In the group of guinea pigs treated with *I. carnea,* we observed anxiety-related behavioural changes; through vocalisation, showing preference for the "small balls" and refusing the fresh alfalfa. It is well-known that goats intoxicated with *Ipomoea* sp. locoweeds, *Swainsona* sp., and *Sida carpinifolia* develop addiction or morbid appetite (Colodel et al., 2002; Ralphs et al., 1990; Riet-Correa and Mendez, 2000). It is also described that animals that consume weeds containing swainsonine induce others to ingest the plant by social facilitation (Ralphs et al., 1994). However, Daló and Moussatché (1978) argued that several species of *Ipomoea* contain lysergic acid derivatives that could be the cause of animal anxiety. Additionally, Pritchard et al. (1990) argued that swainsonine suppresses appetite and retards growth in rats treated with doses over 7.6 mg/kg/day. Thus, the observed preference or addiction to *Ipomoea* leaves in poisoned guinea pigs could be attributed to the derivatives of lysergic acid rather than to the swainsonine, which suppresses the appetite (Pritchard et al. 1990). By contrast, our poisoned guinea pigs ate until the end of the experiment.

The neurologic deterioration in our poisoned guinea pigs, in the forms of neuronal degeneration and

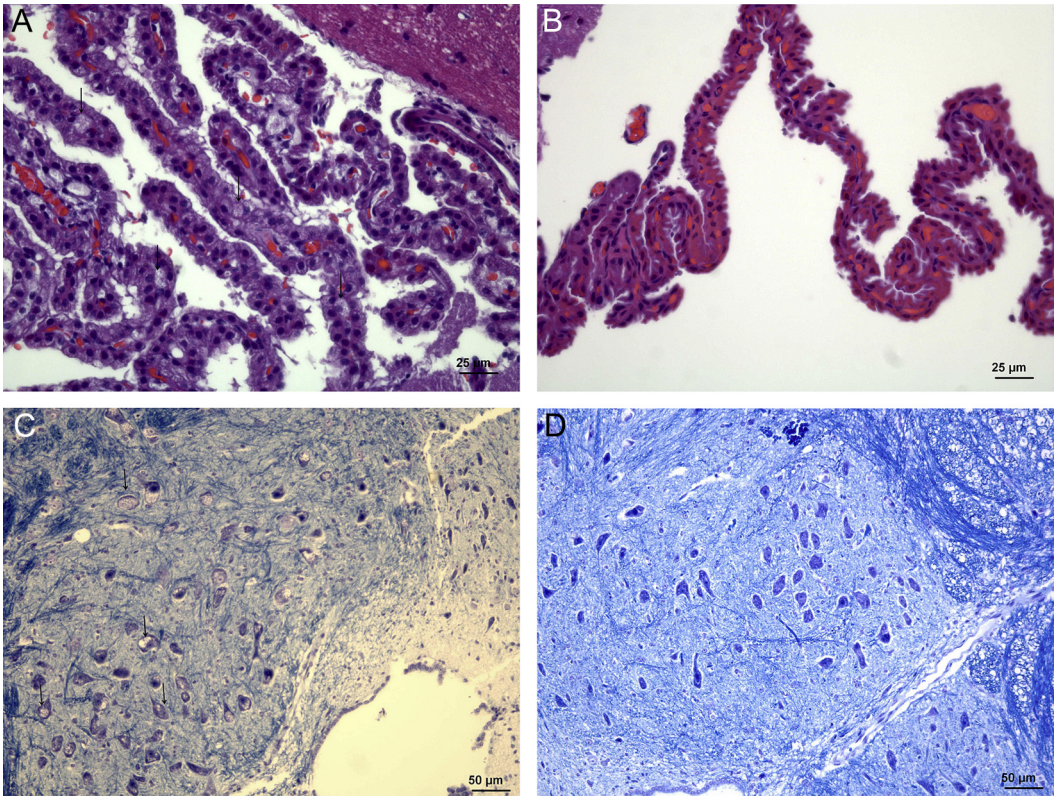

Fig. 2. Light microscopy. Brain. (A) Guinea pigs exposed to "small balls" per 65 days. Choroid plexus epithelium of lateral brain ventricle. Cytoplasmic vacuolation is observed in ependymal cells (arrows), (B) when compared with the control animal. HE stain. Bar, 25 µm. Hypoglossal nucleus in the medulla oblongata. (C) Several neurons with evident vacuoles in guinea pigs exposed to "small balls" per 65 days. (arrows), (D) when compared with the control animal. Kluver Barrera stain. Bar, 50 µm.

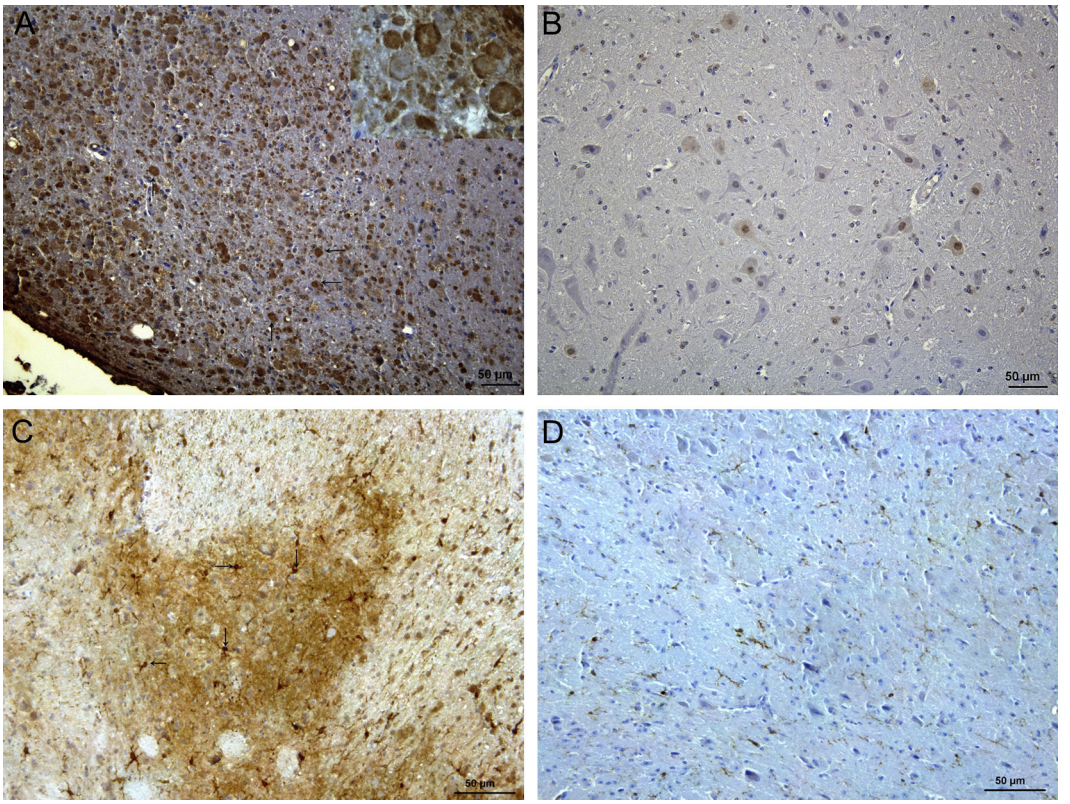

Fig. 3. Immunohistochemical reaction. Brain. Gracile nucleus in the medulla oblongata. (A) Spheroids ubiquitin positive in guinea pigs exposed to "small balls" per 65 days. (arrows). An enlarged view is shown on the right (B) when compared with the control animal. Ubiquitin immunostaining and haematoxylin counterstained. Bar, 50 µm. Gracile nucleus in the medulla oblongata. (C) Microglial cells were stained positively, demonstrating microgliosis in guinea pigs exposed to "small balls" 65 days (arrows), (D) when compared with the control animal. **Iba-1** immunostaining and haematoxylin counterstained Bar, 50 µm.

vacuolisation, was demonstrated by ataxia, lethargy and reduced body condition progressively worsening until the sacrifice. Some of those clinical signs have previously been observed in goats poisoned with the same plant, after 40–65 days of ingestion (Armién et al., 2007; Ríos et al., 2012). Consistent with other authors, a combination of nervous failure and inanition has been suggested to be the cause of the animals' death (Armién et al., 2007). However, in a study in which rats were treated with the different alkaloids and the aqueous fraction from *I. carnea*, the rats presented no clinical symptoms (Hueza et al., 2005). However, mice treated with high doses of swainsonine lost body weight and were clinically anxious, easily excited, and exhibited slight tremors upon movement (Stegelmeier et al., 2008).

The symptoms in guinea pigs suffering congenital α-mannosidosis were first discernable during the first week of life, but the neurological deficits were not obvious before they were two months old (Crawley and Walkley, 2007). Our poisoned animals manifested some of these symptoms beginning in the second week after the onset of the experiment. End-stage clinical disease was observed in congenital α-mannosidosis guinea pigs between 10 and 14 months of age (Crawley and Walkley, 2007). However, extremely poor body condition was observed in our animals at 65 days of intoxication. Therefore, we speculate that the animals poisoned with *I. carnea* would not survive until 10–14 months of age. The difference in survival time between genetic and acquired disease in guinea pigs would be related to the heterogeneity of the toxic components of *I. carnea*. This seems to be in accordance with the findings of some authors, who demonstrated that calystegines act as coadjuvants of swainsonine and that the toxic effects were much more prominent from the aqueous fraction of *I. carnea* (Hueza et al., 2005). Therefore, the toxic compounds of *Ipomoea* may work synergistically to lead to rapid deterioration of the animals intoxicated and to their subsequent deaths. Additionally, swainsonine a is a well-known inhibitor both of lysosomal α-mannosidase and of Golgi mannosidase II (Molyneux et al., 1995; Haraguchi et al., 2003).

The reduced open-field locomotion frequency observed in our intoxicated guinea pigs was similar to those observed in rats exposed to *I. carnea* aqueous extract (Schwarz et al., 2003). The decreased dopamine and serotonin levels observed in our animals would be responsible for the reduced locomotion frequency and paraparesis. The assessment of plasma serotonin concentration is

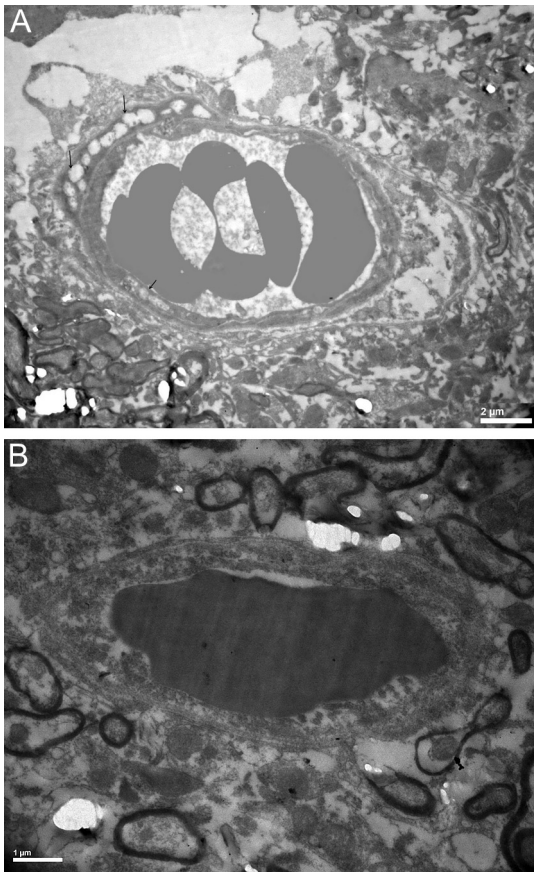

Fig. 4. Electron microscopy. Capillary endothelial cell from brain. (A) Vacuolar appearances of endothelial cells in guinea pigs exposed to "small balls" per 65 days, the vacuoles contain membrane fragments (arrows). Bar, 2 µm (B) when compared with the control animal. Bar, 1 µm.

commonly used to provide information about serotonergic activity in various neurological diseases (Morgadinho et al., 2004). The neuromotor alterations observed in our animals would be a consequence of the neuronal cytoplasmic vacuolation in various areas of the CNS, particularly in the brainstem, where some nuclei are known to be rich in serotonergic neurons (Siegel, 1999). Furthermore, our results are consistent with previous studies suggesting that exposure to *I. carnea* interferes with the serotonergic and dopaminergic systems, which are strongly related to the neuromotor system and to locomotor activity (Airio and Ahtee, 1997; Fink and Smith, 1980; Gimenez-Llort et al., 1997; Jacobs and Fornal, 1995).

Further, the possible presence of lysergic acid derivatives in *I. carnea* would inhibit serotonin at peripheral levels while mimicking serotonin in the CNS, as in the addictive effect observed in animals consuming the plant and weakly antagonising dopamine receptors (Hughes, 1973; Setler et al., 1976). However, the increase of NA observed in our poisoned guinea pigs could be associated with chronic stress caused by the intoxication, as has been previously suggested (Mendels et al., 1972; Setler et al., 1976).

Intraneuronal vacuolation is a classical effect of intoxication by *I. carnea* and other lysosomal storage diseases (de Balogh et al., 1999; Ríos et al., 2008; Stegelmeier et al., 1999). Only relatively high doses of swainsonine produced neurologic histologic changes in mice similar to those observed in goats poisoned with *Ipomoea* sp. and locoweed (Stegelmeier et al., 2008). Rats and mice are relatively resistant to neurologic lesions (Stegelmeier et al., 2007). Surprisingly, guinea pig, though also a rodent, requires much lower doses to develop neuronal vacuolation. In two-month-old guinea pigs with congenital α-mannosidosis, neuronal vacuolation was widespread, and it was severely extensive at seven months of age, except in neurons of the striatum (Crawley and Walkley, 2007). In larger brainstem neurons in particular, we observed numerous vacuoles.

Congenital α-mannosidosis in guinea pigs produces signs of ataxia or motor incoordination beginning at two to three months of age, and these abnormalities are most likely due to various lesions in the nervous system (Robinson et al., 2008). These findings were similar to our gait analysis results. According to Robinson et al. (2008), the gait changes suggest cerebellar dysfunction and spinal cord pathology as potential underlying causes. In our study, only the encephalon was evaluated. Moreover, the vacuole contents were negative for KB and PAS stains, similar to previous studies of α-mannosidosis of different genesis and of swainsonine intoxication, in which vacuoles mainly accumulate incompletely processed oligosaccharides rich in mannose (Hartley, 1971; Van Kampen and James, 1969; Stegelmeier et al., 1999).

Together with the neuronal vacuolation, spheroids were one of the most important findings in the CNS, and they play a key role in the diagnosis and pathogenesis of lysosomal storage diseases, including genetic and acquired α-mannosidosis (Jolly and Walkley, 1997; Walkley, 1998). The spheroids found in our study may be structures corresponding to locally dilated axons that synapse in the gracilis/cuneatus nuclei; similar observations were reported in guinea pigs with congenital α-mannosidosis (Crawley and Walkley, 2007). In agreement with Crawley and Walkley (2007), spheroids in our animals were positive for ubiquitin, but, in contrast, they did not contain calbindin or parvalbumin. These authors labelled ubiquitin, parvalbumin and calbindin to monitor the extent of spheroid formation in congenital α-mannosidosis. The ubiquitin immunostaining of the spheroids would indicate an activation of the nonlysosomal system for the degradation of abnormal filamentous cytoskeletal proteins (Lowe et al., 2001). Calbindin-D28K and parvalbumin are calcium-binding proteins that are considered to be present in spheroids formed in axons of GABAergic neurons. We have no convincing explanation for this difference between the immunohistochemistry of the calcium-binding proteins in congenital and plant-induced α-mannosidosis.

The important neurodegenerative change observed in our animals would be responsible for the propioceptive deficits and paraparesis of affected animals. In fact, the gracilis nucleus is one of the dorsal column nuclei that participate in the sensation of fine touch signals to the cerebral cortex and in proprioception of the hind limb

(Crawley and Walkley, 2007). Huxtable and Dorling (1985) argued that the axonal dystrophic changes observed in rats treated with higher doses of swainsonine for up to 200 days were attributable to normal ageing because the spheroids were also found in untreated rats. In our study, the lesions observed in the gracilis nucleus were attributed to intoxication because our experiment used young animals, and spheroids were absent in the control guinea pigs.

Vacuoles in glial cells were not observed in HE-stained slides. However, glial vacuoles were clearly observed by TEM in our poisoned guinea pigs, in agreement with previous observations reported from goats (Armién et al., 2007). It is likely that the glial functions of providing nutrition and support to neurons were affected. Another relevant finding in this study was moderate microgliosis, as evidenced by expression of Iba-1. Microglia are extremely sensitive to even small pathological changes in the CNS (Dissing-Olesen et al., 2007). The specific functions of microglia are only beginning to be explored (Graeber, 2010). These lesions are similar to previously reported findings from guinea pigs with α-mannosidosis (Crawley and Walkley, 2007). In our animals, apart from vacuoles corresponding to glial cells, neurons and choroid plexuses, vacuoles were also observed under TEM in many capillary endothelial cells, as previously observed in guinea pigs with congenital α-mannosidosis (Auclair and Hopwood, 2007). However, lesions in these additional cell types were not observed in guinea pigs poisoned with *Swainsona galegifolia*, which developed extensive neuronal vacuolation in the central nervous system and peripheral ganglia during a four-week-period (Huxtable, 1970).

Curiously, goat, rats and mice have been preferably used as experimental models to reproduce intoxication by *Ipomoea*, even though guinea pigs provide important advantages because of multiple qualities: despite being rodents, guinea pigs reproduce lysosomal storage diseases at the neuronal level, similar to that which occurs in ruminant and congenital α-mannosidosis. In contrast, rats are a poor model, with the exception of guinea pigs, for reproducing the neurological changes induced by lysosomal storage diseases. This assumption is also based on the result obtained by Stegelmeier et al (1995, 2008) who observed that mice are relatively resistant to swainsonine toxicity. On the other hand, Huxtable and Dorling (1985) demonstrated that rats treated with high doses of swainsonine developed histologic lesions similar to locoism of livestock only in areas of the brain not protected by the blood/brain barrier.

In summary, all of our findings, including cytoplasmic vacuolation in neurons, glial cells, and blood vessel endothelia, as well as axonal spheroids, reveal an extensive neuropathology in a pattern similar to congenital α-mannosidosis. Additionally, behavioural and neurological tests in guinea pigs poisoned with *I. carnea* allowed us to examine the intoxication and measure the relevant monoamine neurotransmitters systematically.

Further studies into the neuropathology in guinea pigs intoxicated with *I. carnea,* such as detailed developmental analysis of the onset and progression of lesions, may be useful for understanding the complex pathological processes of the intoxication and could be related to other acquired and congenital lysosomal storage diseases.

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Conflict of interest Statement

No competing financial interest exists for any of the co-authors of this manuscript.

References

Airio, J., Ahtee, L., 1997. Role of cerebral dopamine and noradrenaline in the morphine-induced locomotor sensitisation in mice. Pharmacol. Biochem. Behav. 58, 379–386.
Armién, A.G., Tokarnia, C.H., Vargas Peixoto, P., Frese, K., 2007. Spontaneous and experimental glycoprotein storage disease of goats induced by *Ipomoea carnea* subsp. *fistulosa* (Convolvulaceae). Vet. Pathol. 44, 170–184.
Auclair, D., Hopwood, J.J., 2007. Morphopathological features in tissues of α-mannosidosis guinea pigs at different gestational ages. Neuropathol. Appl. Neurobiol. 33, 572–585.
Austin, D.F., Huaman, Z.A., 1996. Synopsis of Ipomoea (Convolvulaceae) in the Americas. Taxon 45, 3–38.
Barbosa, R.C., Riet-Correa, F., Medeiros, R.M.T., Lima, E.F., Barros, S.S., et al., 2006. Intoxication by Ipomoea sericophylla and Ipomoea riedelii in goats in the state of Paraíba, Northeastern Brazil. Toxicon 47, 371–379.
Cholich, L.A., Gimeno, E.J., Teibler, P.G., Jorge, N.L., Acosta de Pérez, O.C., 2009. The guinea pig as an animal model for Ipomoea carnea induced α-mannosidosis. Toxicon 54, 276–282.
Colodel, E.M., Gardner, D.R., Zlotowski, P., Driemeier, D., 2002. Identification of swainsonine as a glycoside inhibitor responsible for Sida carpinifolia poisoning. Vet. Hum. Toxicol. 44, 177–178.
Crawley, A.C., Walkley, S.U., 2007. Developmental analysis of CNS pathology in the lysosomal storage disease α-mannosidosis. J. Neuropathol. Exp. Neurol. 66, 687–697.
Daló, N., Moussatché, H., 1978. Acción tóxica de las plantas del género Ipomoea. Tarea Común, Caracas, vol. 6. Univ Centro Occid, pp. 25–39.
de Balogh, K.I.M., Dimande, A.P., Van der Lugt, J.J., Molyneux, R.J., Naudé, T.W., et al., 1999. A lysosomal storage disease induced by *Ipomoea carnea* in goats in Mozambique. J. Vet. Diagn. Invest. 11, 266–273.
Dissing-Olesen, L., Ladeby, R., Nielsen, H.H., Toft-Hansen, H., Dalmau, I., Finsen, B., 2007. Axonal lesion-induced microglial proliferation and microglial cluster formation in the mouse. Neuroscience 149, 112–122.
Dorling, P.R., Huxtable, C.R., Vogel, P., 1978. Lysosomal storage in Swainsona spp. toxicosis: an induced mannosidosis. Neuropathol. Appl. Neurobiol. 4, 285–295.
Dorling, P.R., Huxtable, C.R., Colegate, S.M., 1980. Inhibition of lysosomal α-mannosidase by swainsonine, an indolizidine alkaloid isolated from *Swainsona canescens*. Biochem. J. 91, 649–665.
Driemeier, D., Colodel, E.M., Gimeno, E.J., Barros, S.S., 2000. Lysosomal storage disease caused by Sida carpinifolia poisoning in goats. Vet. Pathol. 37, 153–159.
Fink, J.S., Smith, G.P., 1980. Mesolimbocortical dopamine terminal fields are necessary for normal locomotor and investigatory exploration in rats. Brain Res. 199, 359–384.
Gimenez-Llort, L., Martinez, E., Ferre, S., 1997. Different effects of dopamine antagonists spontaneous and NMDA-induced motor activity in mice. Pharmacol. Biochem. Behav. 56, 549–553.
Graeber, M.B., 2010. Changing face of microglia. Science 330, 783–788.
Haraguchi, M., Gorniak, S.L., Ikeda, K., Minami, Y., Kato, A., et al., 2003. Alkaloidal components in the poisonous plants, *Ipomoea carnea* (Convolvulacea). J. Agric. Food Chem. 51, 4995–5000.
Hartley, W.J., 1971. Some observations on the pathology of *Swainsona* subsp. poisoning in livestock in Eastern Australia. Acta Neuropathol. (Berl.) 8, 342–355.
Hou, J.G., Liu, H.L., He, T.X., Wang, Z.M., Mao, X.F., et al., 2002. Study of the acupuncture effect on monoamine transmitters in rabbit plasma and

brain tissue by high performance liquid chromatography with electrochemical detection. Chin. J. Chromatogr. 20, 140–143.
Hueza, I.M., Guerra, J.L., Haraguchi, M., Naoki, A., Górniak, S.L., 2005. The role of alkaloids in *Ipomoea carnea* toxicosis: a study in rats. Exp. Toxicologic Pathol. 57, 53–58.
Hughes, J., 1973. Inhibition of noradrenaline release by lysergic acid diethylamide. Short communication. Br. J. Pharmacol. 49, 706–708.
Huxtable, C.R., 1970. Ultrastructural changes caused by *Swainsona galegifolia* poisoning in the guinea-pig. Aust. J. Exp. Biol. Med. Sci. 48, 71–80.
Huxtable, C.R., Dorling, P.R., 1985. Mannoside storage and axonal dystrophy in sensory neurones of swainsonine-treated rats: morphogenesis of lesions. Acta Neuropathol. 68, 65–73.
Jacobs, B.L., Fornal, C.A., 1995. Serotonin and behavior. A general hypothesis. In: Bloom, F.E., et al. (Eds.), Psychopharmacology. The Fourth Generation of Progress. Raven Press, New York.
James, L.F., Van Kampen, K.R., Johnson, A.E., 1970. Physiopathologic changes in locoweed poisoning of livestock. Am. J. Vet. Res. 31, 663–672.
James, L.F., Panter, K.E., 1989. Locoweed poisoning in livestock. In: James, L.F., et al. (Eds.), Swainsonine and Related Glycosidase Inhibitors. Iowa State University Press, Ames, Iowa.
James, L.F., Panter, K.E., Gaffield, W., Molyneux, R.J., 2004. Biomedical applications of poisonous plant research. J. Agric. Food Chem. 52, 3211–3230.
Jolly, R.D., Walkley, S.U., 1997. Lysosomal storage diseases of animals: an essay in comparative pathology. Vet. Pathol. 34, 527–548.
Lowe, J., Mayer, J., Landon, M., Layfield, R., 2001. Ubiquitin and the molecular pathology of neurodegenerative diseases. Adv. Exp. Med. Biol. 487, 169–186.
Mendels, J., Frazer, A., Fitzgerald, R.J., Ramsey, T.A., Stokes, J.W., 1972. Biogenic amines metabolites fluid of depressed and maniac patients. Science 20, 1380–1381.
Molyneux, R., Mckenzie, R., O'Sullivan, B., 1995. Identification of the glycosidase inhibitors swainsonine and calystegine B_2 in weir vine *Ipomoea* sp. (aff. *calobra*) and correlation with toxicity. J. Nat. Prod. 58, 878–886.
Morgadinho, M.T., Fontes Ribeiro, C.A., Macedo, T.R., 2004. Influence of the sample preparation method on the serotonin determination in plasma and platelets. Biomed. Chromatogr. 18, 739–744.
Pritchard, D.H., Huxtable, C.R., Dorling, P.R., 1990. Swainsonine toxicosis suppresses appetite and retards growth in weanling rats. Res. Vet. Sci. 48, 228–230.
Ralphs, M.H., Panter, K.E., James, L.F., 1990. Feed preferences and habituation of sheep poisoned by locoweed. J. Anim. Sci. 68, 1354–1362.
Ralphs, M.H., Graham, D., James, L.F., 1994. Social facilitation influences cattle to graze locoweeds. J. Range Manage. 47, 123–126.
Riet-Correa, E., Mendez, M., 2000. Plantas tóxicas e micotoxicoses. Editora e Gráfica Universitaria/UFPEL, Pelotas, Rio Grande do Sul, Brasil.
Ríos, E., Cholich, L., Silva, J., Acosta de Perez, O.C., 2008. Histopathological lesions on central nervous system intoxication of goats by *Ipomoea carnea* spp *fistulosa* (Convolvulácea). Rev. Vet. 19, 130–134.
Ríos, E., Cholich, L.A., Gimeno, E.J., Guidi, M.G., Acosta de Pérez, O.C., 2012. Experimental poisoning of goat by *Ipomoea carnea* subsp. *fistulosa* in Argentina: a clinic and pathological correlation with special consideration on the central nervous system. Pesqui. Vet. Bras. 32, 37–42.
Robinson, A.J., Crawley, A.C., Auclair, D., Weston, P.F., Hirte, C., et al., 2008. Behavioural characterisation of the α-mannosidosis guinea pig. Behav. Brain Res. 186, 176–184.
Rodriguez Armesto, R., Repetto, A.E., Ortega, H.H., Peralta, C.J., Pensiero, J.F., et al., 2004. Intoxicación en cabras por ingestión de *Ipomoea hieronymi* var. *calchaquina* en la Provincia de Catamarca, Argentina. Vet. Argentina 21, 332–341.
Sandoval, E., Barrios, M., Hernández, C., Medina, R., 2010. Study of the daily variation of the derivatives of ergolines in *Ipomoea carnea*. Revista Electrón. Vet. 11, 3. http://www.veterinaria.org/revistas/redvet/n030310/031019.pdf.
Schwarz, A., Gorniak, S.L., Bernardi, M.M., Dagli, M.L., Spinosa, H.S., 2003. Effects of *Ipomoea carnea* aqueous fraction intake by dams during pregnancy on the physical and neurobehavioral development of rat offspring. Neurotoxicol. Teratol. 25, 615–626.
Setler, P., Sarau, H., Mckenzie, G., 1976. Differential attenuation of some effects of haloperidol in rats given scopolamine. Eur. J. Pharmacol. 39, 117–126.
Siegel, G.J., 1999. In: Agranoff, B.W., R, et al. (Eds.), Basic Neurochemistry, Molecular, Cellular and Medical Aspects, sixth ed. Lippincott-Raven, Philadelphia.
Stegelmeier, B.L., Molyneux, R.J., Elbein, A.D., James, L.F., 1995. The lesions of locoweed (*Astragalus mollissimus*), swainsonine and castanospermine in rats. Vet. Pathol. 32, 289–298.
Stegelmeier, B.L., James, L.F., Panter, K.E., Ralphs, M.H., Gardner, D.R., et al., 1999. The pathogenesis and toxicokinetics of locoweed (*Astragalus* and *Oxytropis* subsp.) poisoning in livestock. J. Nat. Toxins 8, 35–45.
Stegelmeier, B.L., Lee, S.T., James, L.F., Gardner, D.R., Panter, K.E., Ralphs, M.H., Pfister, J.A., 2007. Chapter 61. The comparative pathology of locoweed poisoning in livestock, wildlife and rodents. In: Panter, K., Wierenga, T.L., Pfister, J. (Eds.), Poisonous Plants Global Research and Solutions. CABI Publishing, Wallingford, Oxon, UK, pp. 359–365, 665 pp.
Stegelmeier, B.L., Molyneux, R.J., Asano, N., Watson, A.A., Nash, R.J., 2008. The comparative pathology of the glycosidase inhibitors swainsonine, castanospermine, and calystegines A_3, B_2, and C_1 in mice. Toxicol. Patholol. 36, 651–659.
Van Kampen, K.R., James, L.F., 1969. Pathology of locoweed poisoning in sheep. Pathol. Vet. 6, 413–423.
Walkley, S.U., 1998. Cellular pathology of lysosomal storage disorders. Brain Pathol. 8, 175–193.