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The guinea pig as an animal model for *Ipomoea carnea* induced α-mannosidosis

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ABSTRACT

The toxic effects of *Ipomoea carnea* subsp. *fistulosa* were evaluated in guinea pigs by administration of dry leaves during 45 days. Swainsonine and calystegines B_1, B_2 and C_1 were isolated and quantified. Clinical signs included emaciated and loss of body weight. Histological evaluation demonstrates numerous vacuoles in the cytoplasm of pancreas, liver and renal cells. Vacuolation was also evident in neurons of brain stem, mainly pontine nuclei. Neuronal lectin binding pattern showed a strong positive reaction to Con-A (*Concanavalia ensiformis*), WGA (*Triticum vulgaris*), sWGA (succinylated *T. vulgaris*) and LCA (*Lens culinary*). This result is coincident with the lectin histochemistry staining pattern of the vacuoles described in CNS of ruminants. We conclude that *I. carnea* subsp. *fistulosa* induces an intralysosomal accumulation of mannose-containing oligosaccharides in guinea pigs, which makes it a valuable animal model for the reproduction of induced α-mannosidosis.

Induced α-mannosidosis, a lysosomal storage disease resulting from the ingestion of plants of the genera *Swainsona*, *Oxytropis*, *Astragalus*, *Sida*, and *Ipomoea*, has been studied in herbivores in many countries (James et al., 1970; Dorling et al., 1980; James and Painter., 1989; de Balogh et al., 1999; Molyneux et al., 1995; Driemeier et al., 2000; Rodriguez Armesto et al., 2004; Barbosa et al., 2006). These plants contain different polyhydroxylated alkaloids, mainly swainsonine a well known inhibitor of lysosomal α-mannosidase and Golgi mannosidase II (Huxtable et al., 1982; Molyneux et al., 1995; de Balogh et al., 1999; Colodel et al., 2002; Haraguchi et al., 2003). The intoxication induces enzymatic dysfunction and the accumulation of complex oligosaccharides in lysosomes. As a consequence, vacuolation becomes evident in different cells, mainly in neurons (Huxtable et al., 1982; de Balogh et al., 1999; Armién et al., 2007). The clinical syndrome is dominated by neurological and behavioral disorders as well as abnormalities of gait, difficult standing, abnormal posture, emaciation, symmetrical ataxia and posterior limbs paresis (Haraguchi et al., 2003; Tokarnia et al., 2002; Barbosa et al., 2006). Other observed signs are reproductive disorders, abnormal gastrointestinal and immune system functions (Hueza et al., 2003; Armién et al., 2007).

Ipomoea subsp. intoxication has been experimentally induced in ruminants (de Balogh et al., 1999; Barbosa et al., 2006; Armién et al., 2007; Ríos et al., 2007). Mice and rats have been used to study the effects of *Ipomoea* alkaloids (Hueza et al., 2005; Stegelmeier et al., 1995, 2008). Nevertheless, rodents are not good models because no neuronal lesions have been observed in rats (Hueza et al., 2005) or only with very high doses in the case of mice (Stegelmeier et al., 2008).

Although rare in humans, α-mannosidosis is probably the most common and economically important lysosomal storage disease of domestic animals (Jolly and Walkley, 1997). A naturally occurring guinea pig model of this disease, which exhibits a deficiency of lysosomal α-mannosidase, has a similar clinical presentation to human

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inherited α-mannosidosis (Auclair and Hopwood, 2007; Robinson et al., 2008). Prolonged ingestion of pure swainsonine by guinea pigs produced a phenocopy of the genetically induced storage disease α-mannosidosis (Huxtable and Dorling, 1982). The guinea pig has been used as an experimental model only to test the toxicity of *Swainsona galegifolia* (Huxtable, 1969, 1970).

Conventional light microscopic and ultrastructural analysis of tissues serves only to confirm the presence of a lysosomal storage disease and does not generally yield a more specific diagnosis (Jolly and Walkley, 1997). Lectin histochemistry on paraffin-embedded sections may be used to identify specific sugars and hence aids diagnosis of glycoprotein and glycolipid storage diseases (Alroy et al., 1985; Driemeier et al., 2000; Armién et al., 2007; Stegelmeier et al., 2008). Lectins are proteins or glycoproteins of non-immune origin that bind specifically to carbohydrates (Goldstein and Hayes, 1978) and when bound to visulants, allows localization of complementary carbohydrates (Alroy et al., 1985; Damjanov, 1987).

The object of this paper is to report the clinical signs, histological and lectin histochemical analysis of the experimental intoxication by *Ipomoea carnea* dry leaves in guinea pigs.

1. Materials and methods

1.1. Plant identification and toxicological analysis

Leaf samples of *I. carnea* subsp. *fistulosa* were collected from Capital Department, Corrientes Province, Argentina in April 2007. An voucher herbarium specimen was deposited in the Instituto de Botanica, Facultad de Ciencias Agrarias, UNNE (Corrientes, Argentina), under number CTES-395. Taxonomic identification was performed by Taxonomist Roberto Salas of the same Institute.

For toxicological analyses, leaf dry samples of April and November were sent to Dr. Dale Gardner, Poisonous Plants Research Laboratory, in Logan, Utah (Gardner et al., 2001). The leaves used in the experiment were collected in April.

1.2. Experimental designs

Leaves of the plants collected in April were dried at 37 °C up to constant weight and milled. They were mixed homogeneously with commercial guinea pig pellets, previously hydrated with water for making "small balls". The mixture pellet's material–dry leaves in proportion 50:50 was used.

Eight male, 4-week-old, Hartley guinea pigs (80 ± 19 g) were supplied by the Bioterio de la Facultad de Ciencias Veterinarias, UNNE. The animals were housed individually in a temperature controlled room (23 ± 2 °C) and the relative humidity was 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: treated animals ($n = 4$) received the "small balls" of 15 g each and fresh green vegetables *ad libitum*. The control group diet ($n = 4$) consisted of fresh green vegetables and commercial pellets. The intake of *I. carnea* leaves was approximately of 6.25 g/day. The estimate doses of swainsonine and calystegine were 5 mg/kg BW/day and 13.24 mg/kg BW/day respectively. In both groups water was administered *ad libitum*. Through the consumption period, the animals were weighed weekly and feed intake was monitored daily. All of them were sacrificed, previously anesthetized with i.p. injection of chloral hydrate (300 mg/kg), after 45 days of intoxication. The experiment followed the ethical standards for animal experiments in toxicological research recommended by the International Society of Toxicology (Meier et al., 1993).

1.3. Histopathology

Samples were collected immediately after euthanasia for histological examinations. Three different sections were collected in brain, one in cerebellum, three in brain stem, liver, pancreas and kidney. They were fixed in 10% neutral buffered formalin at room temperature, embedded in paraffin, sectioned at 5 μm thickness, stained with hematoxylin and eosin (H&E) and sections of pancreas and liver tissues were stained with periodic acid-Schiff stain to detect carbohydrates and examined under a light microscope.

1.4. Lectin histochemistry

Representative sections of the previously mentioned tissues were submitted to lectin histochemical procedures as follows. Paraffin wax-embedded sections (5 μm) were used. After dewaxing, sections were treated with hydrogen peroxide 0.3% in methanol for 30 min at room temperature (to inhibit the endogenous peroxidase), rinsed several times in 0.01 M PBS (pH 7.2), and treated with bovine serum albumin 0.1% in PBS for 15 min. The sections were then incubated for 1 h at room temperature with biotinylated lectins. Eight lectins (Vector Laboratories, Burlingame, CA, USA) with different specificity were used, as follows: Con-A (*Concanavalia ensiformis*, binding specificity α-D-Man and α-D-Glc); SBA (*Glycine max*, binding specificity α-D-GalNAc, α-D-GalNAc and α and β-Gal); PNA (*Arachis hypogaea*, binding specificity β-D-Gal and (1–3) GalNAc); RCA-I (*Ricinus communis* I, binding specificity β-D-Gal and α-D-Gal); UEA-1 (*Ulex europaeus* I, binding specificity α-L-Fuc); WGA (*Triticum vulgaris*, binding specificity α-D-GlcNAc and NeuNAc); sWGA (*succinyl*-WGA, binding specificity β-(1–4)-D-GlcNAc), and LCA (*Lens culinary*, binding specificity α-Glc, α-D-Man).

The optimal lectin concentration was 30 μg/ml in PBS for all lectins, except PNA, which was applied at a concentration of 10 μg/ml. The slides were incubated with an avidin–biotin–peroxidase complex (ABC) (Vector) for 45 min. The horseradish peroxidase was activated by incubation for 1–2 min with a diaminobenzidine commercial kit (Dako Cytomation, Carpinteria, CA, USA). Specimens were rinsed in distillated water, dehydrated with graded ethanol solutions, cleared in xylene and mounted in Permount (Fisher Scientific International Inc., Liberty Lane Hampton, NH, USA). Controls for lectin staining included: exposure to horseradish peroxidase and substrate medium without lectin; and blocking by incubation with the appropriate blocking sugars (0.1–0.2 M in

PBS) for 1 h at room temperature before applying to the sections. The intensity of lectin binding was subjectively scored from 0 (none) to 3 (strongly positive).

1.5. Statistical analysis

Values were expressed as means ± standard deviation (S.D). Differences among groups were evaluated by using analysis of variance (ANOVA) followed by Tukey test. The level of significance was set at $p < 0.05$.

2. Results

2.1. Identification of the plant

The plant was identified as *I. carnea* subsp. *fistulosa* from the Convolvulaceae family, known in Northern Argentina with the common guarani names of "mandiyurá" or "aguapeí".

2.2. Identification of the active principle of the plant

Polyhydroxy alkaloids were detected as chemical constituents of plants, swainsonine, calystegine B_1, calystegine B_2, calystegine C_1, and trace amounts of calystegine A_3 and B_3 (Fig. 1). Swainsonine concentration in April 2007, and given to the experimental guinea pigs, was of 0.02% and in November of 0.01%, whereas the mean contents of Calystegine was 0.053% in April and of 0.046% in November.

2.3. Clinical signs

At the very beginning of the study, the animals refused to eat the "small balls", after a week they accepted it. Food intake in the experimental animals was reduced around 50% in comparison with the controls. One week after the beginning of the experiment the animals were reluctant to move and indifferent. The clinical signs become accentuated after 30 days of intoxication. The administration was suspended after 45 days; the animals were euthanized immediately after the presentation of severe intoxication clinical signs. The animals showed hirsutism, progressively emaciated and body weight gain was lower in the animals poisoned ($^1p < 0.0001$) compared to values of controls (Fig. 2). At necropsy, no significant macroscopic lesions were observed in the animals studied.

The control animals did not show any clinical signs or abnormalities in feed intake during the experiment. No difference in water consumption was detected between groups.

2.4. Microscopic lesions

Histopathological evaluation of treated animals staining with H&E, revealed the presence of small cytoplasmatic vacuoles in hepatocytes, Kupffer cells, exocrine pancreas and renal tubular epithelium cells (Fig. 3B, D). Neurons of all parts of the brain stem, mainly neurons of the pontine nuclei, were swollen and distended by vacuolation of the pericaryon (Fig. 3F). On the other hand, the vacuoles present within liver, pancreas and kidney were negative for carbohydrates by PAS stain. The controls did not show any significant lesions (Fig. 3A, C, E).

2.5. Lectin histochemical findings

The results of the lectin binding patterns for affected and control animals are summarized in Table 1. The vacuolated neurons were strongly stained with Con-A, sWGA, WGA and LCA (Fig. 4D). These cells did not stain in corresponding slides from control animals (Fig. 4C).

These results are similar to the staining pattern of the vacuoles detected in pancreas, liver and kidney (Fig. 4B), except for sWGA which reacted only with vacuolated neurons. Additionally, a partial reaction of pancreatic, liver and kidney cells to UEA-I, RCA-I, SBA was observed in affected and control tissues. Furthermore, PNA did not react with vacuolated neurons but it did in the cells in other organs.

3. Discussion

This experimental study demonstrated that the intake of 50% of dry leaves of *I. carnea* during 45 days induces a lysosomal storage disease in guinea pigs. In the present study a mean dose of 5 mg of swainsonine per Kg body weight per day and a mean dose of 13.24 mg of calystegine/Kg BW/day were used.

Cytoplasmatic vacuolation was evident in cells of liver, exocrine pancreas, kidney, medulla oblongata and pons. The lesions are similar to the acquired α-mannosidosis secondary to the ingestion of plants of the genera *Swainsona*, *Oxytropis*, *Astragalus*, and *Ipomoea* by herbivores. The mechanism involved in α-mannosidosis is a deficiency of lysosomal α-mannosidase, an enzyme that catabolizes the various moieties of glycoproteins. As a consequence,

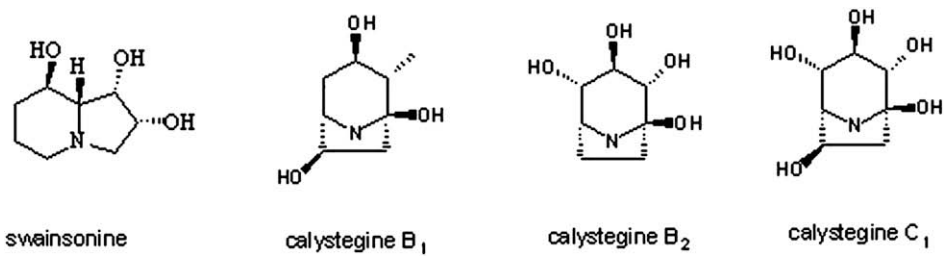

Fig. 1. Structures of the Polyhydroxy alkaloids found in the plant *Ipomoea carnea* from Corrientes, Argentina.

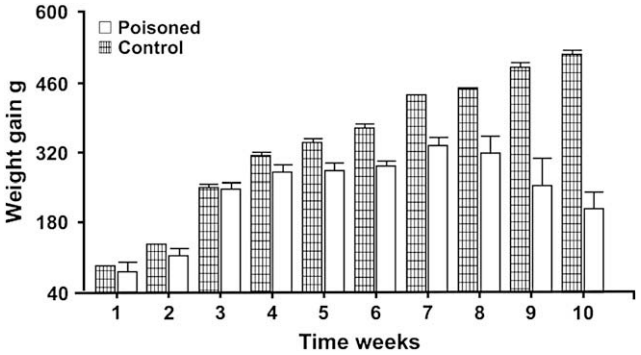

Fig. 2. Body weight gain in guinea pigs during 45 days. The three first weeks corresponding to adaptation and weights homogeneity of animals group. $p < 0.0001$ when compared to values of controls.

a variety of mannose-containing oligosaccharides derived from high-mannose complex, and hybrid oligosaccharides accumulate in lysosomes (Jolly and Walkley, 1997).

The initial isolation of swainsonine from *Swainsona* species by Colegate et al. (1979) resulted from the recognition that the poisoning induced in livestock by these plants was biochemically, morphologically, and clinically similar to the genetic disease mannosidosis, which results from an insufficiency of the enzyme α-mannosidase. However, Swainsonine also inhibits Golgi mannosidase II, a key enzyme in the glycosylation of many glycoproteins. As a consequence, a variety of mannose-containing oligosaccharides derived from high-mannose, complex, and hybrid oligosaccharides accumulate in lysosomes (Dorling et al., 1978; Jolly and Walkley, 1997).

The congenital mannosidosis is inherited and genetically determined. Clinical signs appears early in life and the progression is irreversible (Robinson et al., 2008). This acquired storage disease mimics the inherited α-mannosidosis of domestic animals described principally in Angus cattle in New Zealand, Australia, Scotland, North America, Argentina and Uruguay due to a deficiency of lysosomal α-mannosidase, an enzyme that normally catabolizes the various oligosaccharide moieties of glycoproteins (Jolly and Walkley, 1997; Rivero et al., 2001).

In our investigation, the alkaloids isolated and identified from the dry leaves of *I. carnea* were swainsonine and calystegines B_1, B_2 and C_1. These alkaloids have been isolated from the same plant from other regions of the world (de Balogh et al., 1999; Haraguchi et al., 2003). Swainsonine was first isolated from *Swainsona* subsp. and characterized as a potent and specific inhibitor of the enzyme α-mannosidase (Gardner et al., 2003). This alkaloid has also been reported in other plant-species in Australia, United States, Mozambique and Brazil (Stegelmeier et al., 1999; de Balogh et al., 1999; Colodel et al., 2002; James et al., 2004).

The concentrations of alkaloid contents in dry leaves were significantly different to that reported from Brazil by Haraguchi et al. (2003), where the amount of alkaloids in fresh leaves was 0.0029% for swainsonine, 0.0007% and 0.0021% for calystegine B_1, B_2, respectively. Molyneux et al. (1995) suggested that levels in excess of 0.001% are probably toxic. Also, we coincident with Armién et al. (2007), the variation in quantities of toxin contained neither affected the disease progression nor the degree of histopathologic lesions.

Signs of toxicity were first observed 30 days after the beginning of the experiment. The observed clinical features of experimental *I. carnea* poisoning were similar to observations in rats and included anorexia and a subsequent lost body mass (Hueza et al., 2003). An hypothesis is that the weight loss in guinea pigs over the course of the study could be a result of reduced enzymatic digestion, neurological deficits, or both, because swainsonine inhibits digestive enzymes such as sucrase (Pan et al., 1993), and this inhibition likely reduces digestive efficiency and supports weight loss.

In the present study, neuronal vacuolation was found mainly at pontine nuclei. Huxtable (1969) reported that area as the more affected in *S. galegifolia* intoxicated guinea pig.

Comparative studies in rats, mice, hamsters, mule deer, sheep and cattle with known quantities of swainsonine and locoweed showed major interspecies differences regarding the sensitivity. Horses were the most sensitive, cattle and sheep have similar sensitivity and mule deer and rodents were considered relatively resistant to poisoning (Stegelmeier et al., 2007). Nevertheless, vacuolation of cortical neurons have been observed in mice receiving very high doses of swainsonine (Stegelmeier et al., 2008). Huxtable and Dorling (1985) gave swainsonine in drinking water to rats for periods up to 200 days; they have observed neuronal mannoside storage only in peripheral ganglia and in those areas of the brain not protected by the blood/brain barrier. On the other hand, a similar dose in guinea pigs leads to the development of extensive neuronal vacuolation in the central nervous system and peripheral ganglia in four weeks (Huxtable and Dorling, 1982).

Other studies also indicate that the central nervous system is protected against swainsonine by an extensive barrier of astrocytes and endothelial cells in mice (Bowen et al., 1993). Our results using dry leaves of *I. carnea*, seem to indicate that the guinea pig is highly susceptible to the mixture of alkaloids present in the plant. At the present time, there is no explanation for the resistance of rats, mice and hamsters. Additional work will be required to explain why the guinea pig, being also a rodent, is susceptible.

Our study also revealed the nature of stored material in lysosomal vacuoles using lectin histochemistry. The pattern

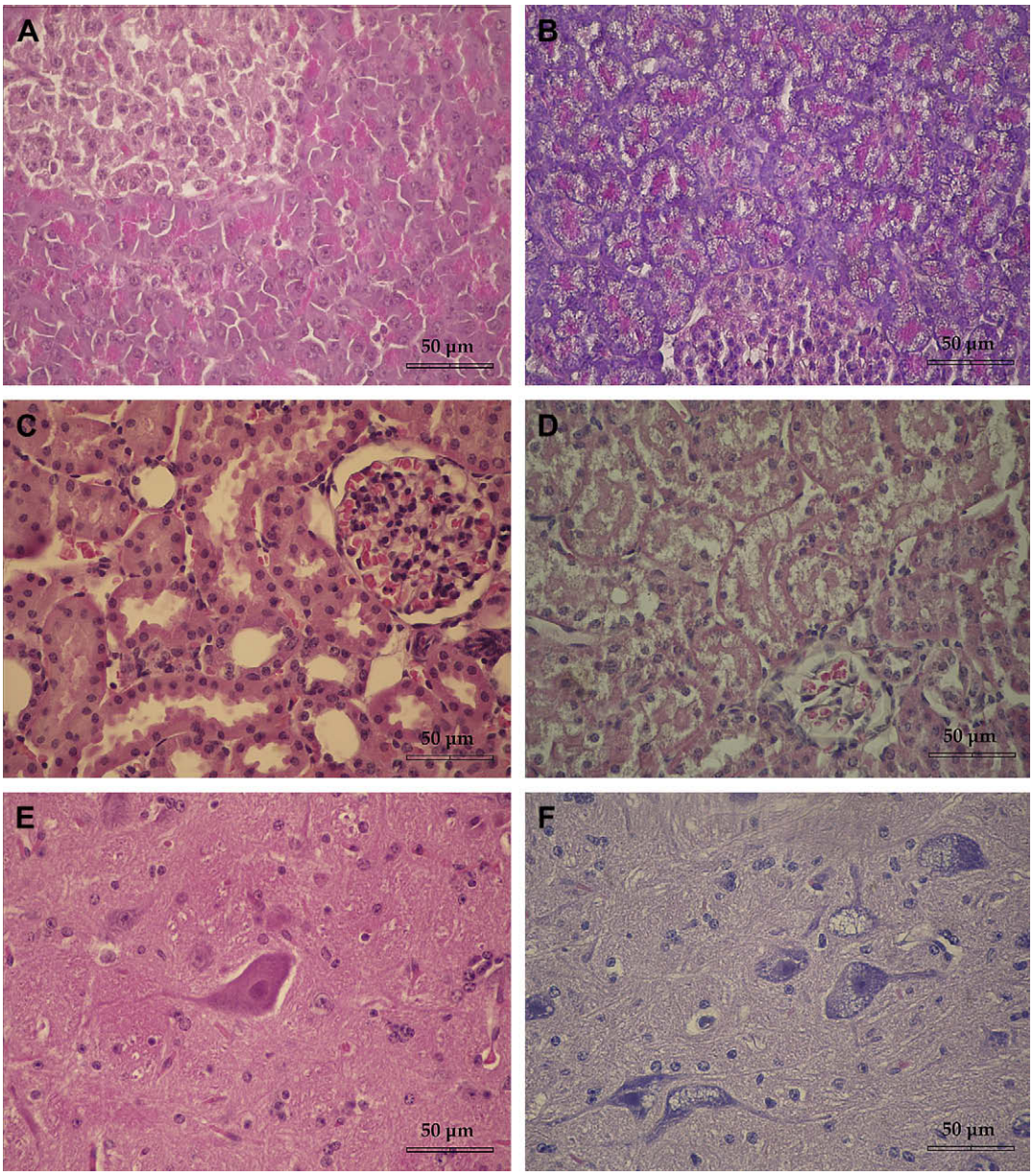

Fig. 3. Appearance of organs in control and *I. carnea*-intoxicated guinea pigs. Normal pancreas (A), kidney (C) and pons (E). In affected animals vacuoles are seen in epithelium of exocrine pancreas (B) and kidney (D). Neurons at the pontine nuclei showed clear perikaryal vacuolation (F). H&E. Scale bar, 50 µm.

of lectin staining observed in neurons partially agrees with the results reported for locoweed and Swainsonine toxicosis and for mannosidosis in humans, cats, and calves (Alroy et al., 1985). In feline mannosidosis, WGA and Con-A recognized the undegraded glycoproteins and oligosaccharides stored in lysosomes of affected cells as was the case in *I. carnea* intoxication (Castagnaro, 1990). The reaction was clear to sWGA, WGA which indicates the accumulation of β-D-*N*-acetyl-glucosamine and *N*-acetyl-neuraminic acid, and Con-A, specific for α-D-mannose and α-D-glucose (Goldstein and Hayes, 1978). The neuronal reaction to LCA is in agreement with the findings of Armién et al. (2007) and is a clear indication of the presence of α-glucose and α-D-mannose. This result is coincident with the staining pattern of the vacuoles found in pancreas, liver and kidney, except for sWGA. Also in *Ipomoea*-intoxicated goats, LCA reacted strongly in nervous tissues and negatively to very mild in liver, kidney and pancreas (Armién et al., 2007).

The partial reaction of pancreatic, liver and kidney cells with UEA-I (*U. europaeus* I), RCA-I (*R. communis* I), SBA (*G. max*) was observed in affected and control guinea pigs.

The exocrine acinar cells in pancreas of affected guinea pigs were positive to WGA, Con-A, PNA and LCA which is

Table 1
Intensity of lectin binding on affected cells in organs evaluated from poisoned and normal guinea pigs.

Cell type	Lectins							
	Con-A	sWGA	LCA	PNA	RCA-I	SBA	UEA-I	WGA
Exocrine pancreas	3 (0)[a]	0 (0)	3 (1)	3 (1)	1 (2)	1 (2)	1(2)	3 (1)
Hepatocytes	2 (0)	0 (0)	2 (0)	0 (0)	1 (1)	0 (0)	1 (0)	0 (0)
Kupffer cells	0 (0)	0 (0)	3 (1)	2 (1)	1 (2)	0 (0)	1 (0)	2 (0)
Kidney tubular epithelium	2 (0)	0 (0)	2 (1)	3 (1)	1 (1)	1 (1)	0 (0)	3 (1)
Neurons of brain stem nuclei	3 (1)	3 (0)	3 (1)	0 (0)	0 (0)	0 (0)	0 (0)	3 (1)

[a] Numbers indicate staining intensity on a subjective estimated scale from 0, unreactive to 3, most reactive. Control results from normal guinea pigs are provided in the parenthesis.

partially coincident with previous reports (Driemeier et al., 2000; Armién et al., 2007). The adult exocrine acinar cells of mouse, rats, guinea pigs and rabbits expressed at a more or less high rate of binding of PNA, Con-A, UEA-I, RCA-I, and WGA (Muresan et al., 1982). The lectin binding properties revealed extensive and specific heterogeneity of acinar cell population; a population which appears homogeneous by classical light and electron microscopic preparations (Jeraldo et al., 1996). The partial staining obtained in our study with UEA-I, RCA-I and SBA corresponds to the lectin binding pattern of normal acinar cells in goats; this was corroborated by the lectin reactivity of control guinea pigs.

We conclude that *I. carnea* subsp. *fistulosa* induces a glycoprotein storage disease in guinea pigs, which makes it a valuable animal model. The discovery of a novel model for plant induced α-mannosidosis opens a wide array of possibilities for further studies. For instance, the striking differences in susceptibility observed when comparing the guinea pig with other rodents. It could also be a model superior than rats and mice for toxicology evaluation of individual alkaloids.

Goats have been extensively used as a model to study plant induced α-mannosidosis (de Balogh et al., 1999; Driemeier et al., 2000; Barbosa et al., 2006; Stegelmeier et al., 2007). The present guinea pig model could be an easier and cheaper alternative. It remains to be determined if the guinea pig is sensitive to other swainsonine containing plants, namely *Oxytropis* subsp., *Astragalus* subsp., *Ipomoea* subsp., and *Sida carpinifolia*.

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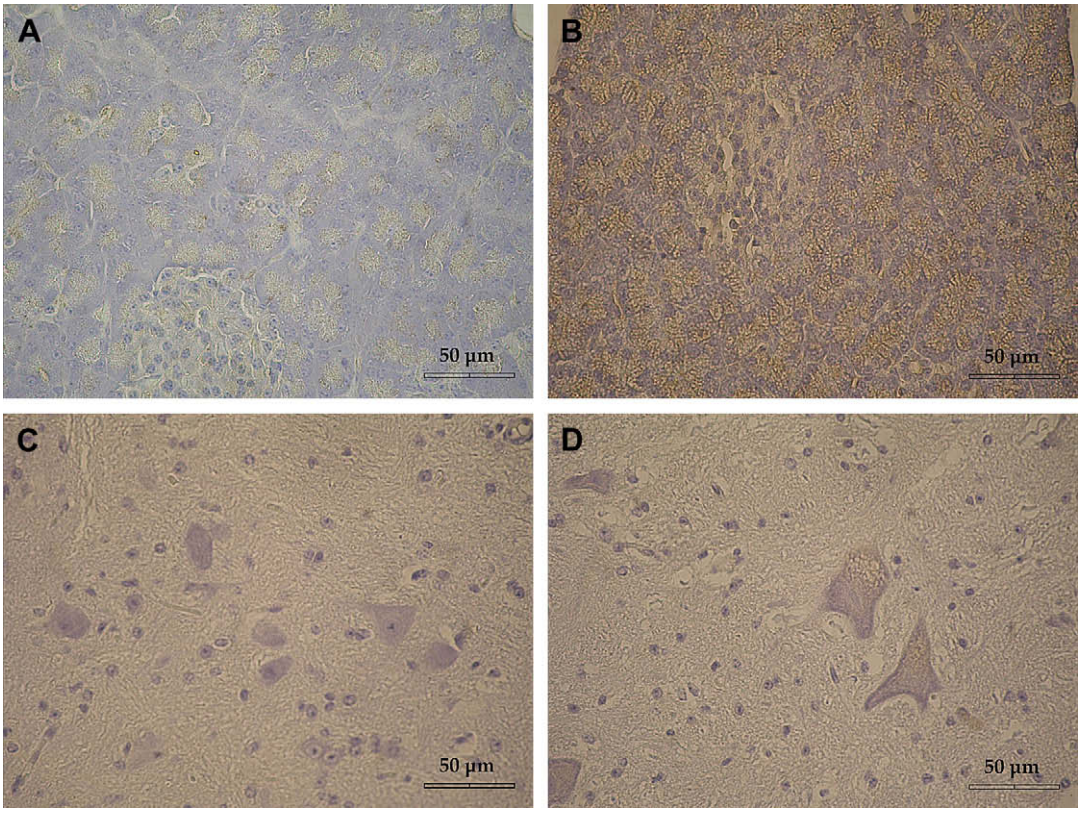

Fig. 4. Lectin histochemistry. In affected animals the exocrine pancreas is positive for WGA (B) and pons stains positive with sWGA (D). In control animals pancreas (A) and neurons (C) are negative for the same lectins. Avidin–Biotin Complex method. Scale bar, 50 µm.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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