Toxicon 190 (2021) 3–10

Contents lists available at ScienceDirect

Toxicon

journal homepage: http://www.elsevier.com/locate/toxicon

The effect of *Ipomoea carnea* on maternal reproductive outcomes and fetal and postnatal development in rats

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ARTICLE INFO

Keywords:
Ipomoea carnea
Swainsonine
Reproductive toxicology
Placental toxicity
Birth defects

ABSTRACT

Ipomoea carnea is a toxic plant found in Brazil and other tropical countries. The plant contains the alkaloids calystegines and swainsonine, which inhibit key cellular enzymes and cause systematic cell death. It is known that swainsonine is excreted in the amniotic fluid of dams exposed to the plant. Thus, the aim of this study was to verify whether the toxic effect of *I. carnea* on fetuses is due to exclusively the passage of the active principle of the plant through the placenta, or if the placentotoxic effect of swainsonine could collaborate in the adverse effects observed in the fetus. The teratogenic effects of exposure to the toxic principles of *I. carnea* were evaluated not only using the conventional protocol but also at later stages in the postnatal developmental period. Females were treated, from gestation day (GD) 6 until GD19, with 0.0, 1.0, 3.0 or 7.0 g/kg body weight of *I. carnea* dry leaves. The plant did not induce changes in reproductive performance or biochemical profile of the dams. Dams that received the highest dose of *I. carnea* showed cytoplasmic vacuolization in the liver, kidney and placental tissue. *I. carnea* promoted different lectin binding patterns in different areas of placental tissue. No fetal skeletal or visceral malformations was observed. The postnatal evaluation revealed a lower litter weight and a lower pup body weight one day after birth in the group that received the highest dose of *I. carnea*. Physical milestones were unaffected by the treatments. Female pups from all experimental groups exhibited a delay in achieving a negative geotaxis response. The results show that the toxic principle of *I. carnea* produces injury *in utero* in mothers and fetuses, but these deleterious effects were better demonstrated using postnatal evaluation.

1. Introduction

Ipomoea carnea Jacq. spp. fistulosa Choisy (Convolvulaceae) is a poisonous plant widely distributed in Brazil (Tokarnia et al., 2000) and other tropical countries (Austin and Huáman, 1996). Natural toxicosis occurs when different farm animal species chronically ingest the plant; after prolonged periods of plant intake, the animals exhibit a variety of clinical signs, such as depression, general weakness, body weight loss, staggering gait, muscle tremors, ataxia, posterior paresis, and paralysis (Damir et al., 1987; De Balogh et al., 1999; Tokarnia et al., 2000; Schumaher-Henrique et al., 2003).

Intoxication induces enzymatic dysfunction, which causes the accumulation of complex oligosaccharides in lysosomes. As a consequence, vacuolation becomes evident in different cells, mainly in the CNS (De Balogh et al., 1999; Schumaher-Henrique et al., 2003; Gotardo et al., 2012).

Two kinds of toxic components were isolated from the plant, the nortropane alkaloids calystegines B1, B2, B3 and C1 and the most important active principle, the indolizidine alkaloid swainsonine (Molyneux et al., 1995; De Balogh et al., 1999; Haraguchi et al., 2003). Swainsonine is a potent inhibitor of two distinct intracellular enzymes, the lysosomal α-mannosidase, which results in lysosomal accumulation of incompletely processed oligosaccharides rich in a-mannosyl and b-N-acetyl glucosamine moieties inside vacuoles, which progresses to cellular function loss and, ultimately, to cell death (Tulsiani and Touser, 1983), and the Golgi mannosidase II enzyme, which causes alteration of

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https://doi.org/10.1016/j.toxicon.2020.11.012
Received 2 October 2020; Received in revised form 19 November 2020; Accepted 22 November 2020
Available online 27 November 2020

the N-linked glycoprotein process, modifying glycoprotein synthesis, processing and carrier (Moremen and Robbins, 1991). Histologically, cellular vacuolization of Purkinje cells, thyroid follicles, exocrine pancreas, liver, kidney and testis cells has been observed (Hueza et al., 2005; Gotardo et al., 2012, 2014).

Many studies in ruminants (De Balogh et al., 1999; Pfister et al., 2006; Antoniassi et al., 2007) have shown that *I. carnea* intake over a prolonged period of time induces neurobehavioral effects, and we verified that offspring of pregnant goats given *I. carnea* during gestation have significant behavioral alterations and developmental delays (Gotardo et al., 2011, 2016). In addition, a study conducted in our laboratory with pregnant rats showed that swainsonine was found in mother's serum and amniotic fluid (Hueza et al., 2007).

The placenta is a temporary extraembryonic organ with various functions that provides the connection between the fetus with the parent organism; thus, during the prenatal period, the placenta is the essential interface for the exchange of oxygen, nutrients, and metabolic waste between the mother and fetus. Moreover, the placenta becomes the major source of hormones necessary for pregnancy maintenance, parturition, and maternal and fetal health (Roescher et al., 2014). Hence, placental injury could lead to dysfunction, producing adverse effects on fetal growth and development (Ilekis et al., 2016). Thus, one of the two aims of this study is to verify whether the toxic effect of *I. carnea* on fetuses is due to exclusively the passage of the active principle of the plant through the placenta, causing injury directly to the conceptus, or if there is also a placentotoxic effect of swainsonine, which could work together in the adverse effects observed in the fetus.

There is a great concern regarding the accuracy of the existing protocols to check the potential exposure risks of the chemical substances during fetal development, with one of the most important limitations of these protocols being the lack of examination of the offspring during the postnatal period. Therefore, the second goal of the present study is to better evaluate the toxic effects of *I. carnea* to dams exposed to the plant during the organogenesis and fetal development periods of gestation, verifying the possible teratogenic effects of exposure to the toxic principles of *I. carnea* not only using the conventional protocol (i.e., assessing developmental effects in fetuses just before term) but also at later stages in the postnatal developmental period.

2. Materials and methods

2.1. Plant material

I. carnea leaves were collected from plants cultivated at the Research Centre for Veterinary Toxicology (CEPTOX), University of São Paulo (USP), Pirassununga, Brazil (21°58′ S, 47°27' W). A voucher specimen was deposited in the Herbarium of the Instituto de Botânica de São Paulo, Brazil (SP-360911), and its authenticity was confirmed by a taxonomist of the same institute.

The aqueous fraction (AF) resulting from the extraction of dry *I. carnea* leaves was obtained according to previously described methods (Hueza et al., 2003). Briefly, dry leaves were macerated in 96% ethanol. After total solvent evaporation under reduced pressure at 50 °C, a dark green extract was obtained, which was suspended in water to remove the waxy residue and consecutively fractionated with *n*-butanol saturated with water. The remaining aqueous solution was lyophilized to give an AF.

To prepare the doses used in the present study, distilled water was added to the AF to achieve a mixture with a final concentration of 1, 3 and 7 g/ml of *I. carnea* dry leaves for use.

2.2. Swainsonine and calystegine contents

A representative sample of AF was collected at the beginning of the study and used for analysis. The swainsonine and calystegine concentrations were determined on several subsamples of this sample using reversed-phase high-performance liquid chromatography coupled to atmospheric pressure chemical ionization tandem mass spectrometry (LC-MS2 (Finnigan LCQ ITE, Mundelein, IL, USA)). The quantitation limit (i.e., detection limit) was estimated to be 0.001% for swainsonine (Gardner et al., 2001); no limit has been determined for calystegines.

2.3. Animals, feeding and housing

Eighty-four pregnant Wistar Hanover rats (90 days of age) were produced in our own colony (Department of Pathology, School of Veterinary Medicine, University of São Paulo). Gestational day (GD) 0 was defined as the day when spermatozoids were detected in the vaginal lavage.

All rats were housed separately in polycarbonate cages measuring 40 × 50 × 20 cm, and the cage bottoms were lined with sterilized wood shavings under controlled conditions of temperature (22–25 °C), relative humidity (50–65%) and lighting (12/12-h light/dark cycle). The animals were provided with commercial rodent food (Nuvilab Nuvital-CR1®) and fresh water *ad libitum*. Clinical observations were performed every other day to evaluate signs of intoxication and mortality as proposed by the Organisation for Economic Co-operation and Development protocols (Test No. 414: Prenatal Developmental Toxicity Study; OECD, 2018).

2.4. Experimental design

Pregnant rats were randomly allocated into four treatment groups and given daily, from GD6 until GD19, the following doses of *I. carnea* dry leaves present in the AF, in g per kg of body weight (g/kg BW), by gavage: 0 (control group, n = 21), 1.0 (IC1 group, n = 21), 3.0 (IC3 group, n = 21), and 7.0 (IC7 group, n = 21). Food and water consumption and body weight (BW) were measured every three days during the gestational period.

2.4.1. Maternal reproductive outcomes and fetal development

At GD20, 11 dams from each group were anesthetized with intraperitoneal injections of xylazine and ketamine (5 and 50 mg/kg, respectively) to prepare for cesarean section. The gravid uterus weight and the number of *corpora lutea*, implantations, and resorptions were recorded. The fetuses were removed from the uterus, weighed, and sexed, and the fetal length was measured. The viability was evaluated immediately after birth, and the numbers of live and dead fetuses were recorded. The placental weight was also measured.

After this procedure, alternate fetuses were randomly selected for either skeletal examination (4 fetuses/litter for a total of 44 fetuses/group) according to the technique of Staples and Schnell (1964) or for visceral examination (4 fetuses/litter for a total of 44 fetuses/group) by the Wilson (1965) method using serial sectioning. The fetal observations were recorded in accordance with internationally developed terminology (Wise et al., 1997).

Blood samples were collected from the hepatic vein for serum biochemical analyses. Commercial kits (CELM®, Barueri, São Paulo, Brazil) were used to determine the levels of alkaline phosphatase (AP), alanine transaminase (ALT), aspartate transaminase (AST), glucose, γ-glutamyl transpeptidase (GGT), cholesterol, urea, creatinine, total protein and albumin using a CELM SBA-200 biochemistry analyzer (CELM®). Tissue samples of the CNS, liver, kidney, pancreas, thyroid, lung and placenta were also collected and fixed in 10% formalin, routinely embedded in paraffin, cut into 5-μm-thick sections and stained with hematoxylin and eosin (HE) for histopathology. Morphometry analyses were also performed with image analysis software (Image-Pro Plus® -Media Cybernetics Inc., Bethesda MD, USA).

Lectin histochemistry was performed in placental tissues from the control and IC7 groups using six lectins (Vector Laboratories Inc. Burlingame, Ca, USA), with different specificities: *Arachis hypogaea* (PNA; β-D-galactose(1–3)N-acetylgalactosamine), *Bandeira simplicifolia* (BS-;

α-D-galactose), *Dolichos biflorus* (DBA; α-D-N-acetylgalactosamine), *Ricinus communis* I (RCA-I; β-D-galactose), *Triticum vulgaris* (WGA; β-D-N-acetylglucosamine), and succinyl-*T. vulgaris* (S-WGA; (β-(1–4)-D-N-acetylglucosamine)^2). After deparaffinization, they were hydrated in an alcohol series following incubation in 3% hydrogen peroxide in methanol for 30 min at room temperature to block endogenous peroxidase. Afterward, the slides were rinsed 3 times with 0.01 M phosphate-buffered saline (PBS), pH 7.2, for 3 min each. Then, the slides were treated with 0.1% bovine serum albumin in PBS for 30 min prior to incubation with lectins. The sections were then incubated with biotinylated lectins at room temperature for 2 h. Each lectin was washed separately, 3 times for 3 min each, with 0.01 M PBS. After the lectins were washed, they were incubated with streptavidin-peroxidase complex (R.T.U.; Vector Laboratories Inc. Burlingame, Ca, USA) for 30 min at room temperature. The chromogen system with diaminobenzidine, Liquid DAB® (Dako North America Inc. Carpinteria, Ca, USA), was used to stain the lectins in the tissue slides for 30 s, at which point the brownish color developed on the positively marked slides. All sections were counterstained with Mayer's hematoxylin. Each lectin was used at a dilution of 40 μg/ml with 1% bovine serum albumin in PBS, except for PNA, which was applied at a concentration of 20 μg/ml. The reactivity to lectins was estimated by the reactivity score and arranged on a scale of -(0) unreactive, +(1) discrete, ++(2) moderate, and +++(3) intense.

Apoptotic cells of the placental tissue from the control and IC7 groups were quantified by TUNEL assay using a specific kit (ROCHE *In Situ* Cell Death Detection Kit, Fluorescein) following the manufacturer's recommendations. The percentage of apoptotic cells was calculated from the count of 1000 cells in the junctional zone of placental tissue per slide using image analysis software (Image-Pro Plus® -Media Cybernetics Inc, Bethesda MD, USA).

2.4.2. Postnatal evaluation

Ten dams from each group were allowed to give birth and nurture their offspring normally. Delivery day (postnatal day – PND) was defined as PND0. On PND1, all the litters were externally examined and sexed, and the total litter weight was recorded. Litters were culled to obtain four males and four females from each litter, leaving each mother with eight pups.

One male and one female pup of each litter (ten males and ten females/group) were randomly selected and marked on PND1. Before weaning (PND1-PND21), an experimenter blinded to the animal's treatment condition tested marked rats from all litters for physical, neuromotor and reflex development. Pups were observed daily at the same time and separated from the mothers only at the time of the test. On PND1, 7, 14 and 21, marked male and female pups were weighed.

For the assessment of physical development milestones, the following details were observed and recorded: hair growth, pinna detachment, incisor eruption, opening of the auditory canal, eye opening and adult gait. Responses to the following neuromotor and reflex development were measured: surface righting reflex (pup's ability to turn over from a supine position at surface level; each pup was tested three times in each day with the maximum time allowed for each test of 30 s); negative geotaxis (the pup's ability to turn 180° on a 25° incline placed head down; each pup was tested three times in each day with the maximum time allowed for each test of 30 s) and startle reflex (the presence or absence of sensorimotor reaction to auditory stimulus). The evaluated parameters were expressed as the cumulative number of days required for the appearance of these milestones.

On PND 7, 14, and 22, one pup of each mother was euthanized by cervical dislocation. Tissue samples of the CNS, liver, kidney, pancreas, thyroid and lung were collected and fixed in 10% formalin, routinely embedded in paraffin, cut into 5-μm-thick sections, and stained with HE for histopathology. The same procedure was performed for the mothers on PND22.

2.5. Statistical analysis

The data were analyzed using GraphPad Prism 6.01® software (GraphPad Software, Inc., San Diego, CA, USA). The homoscedasticity was verified by the F or Bartlet test. Normality was checked by the Brow-Forsythe test. One-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons *post hoc* was used to analyze food and water consumption, body weight gain, reproductive end points, biochemical data, skeletal and visceral examination, physical and neuromotor development. Nonparametric data from placental morphometry and apoptotic cells acont of the placenta were compared by the Kruskal–Wallis test, followed by Dunn's test. Data are expressed as the mean ± standard error of the mean (SEM), and differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Swainsonine and calystegine contents

The AF of *I. carnea* dry leaves contained 0.14% swainsonine based on the dry weight of the leaves. Thus, it was calculated that pregnant females from the different groups ingested the following daily doses of swainsonine, in mg/kg BW: control = 0.0; IC1 = 0.27; IC3 = 0.81; and IC7 = 1.89. The calystegine concentrations in the plant were 0.03%, 0.07%, and 0.03% for calystegines B1, B2, and C1, respectively.

3.2. Maternal reproductive outcomes and fetal development

None of the females in the different groups showed any clinical changes during the gestational period. Food and water consumption and reproductive performance of the dams treated with the different doses of *I. carnea* AF from the 6th to the 19th days of gestation were unaffected by the treatment (Table 1, $p > 0.05$). A lower body weight gain was observed in the group that received the highest dose compared to the control group (Table 1, $p < 0.05$). Serum biochemical analyses in the dams showed discrete fluctuations; however, the values remained

Table 1
Food (g) and water (mL) consumption, reproductive performance and fetal measurements (mean ± S.E.M.) of pregnant rats treated by gavage with different doses of *I. carnea* aqueous fraction (AF) from days 6–19 of gestation (n = 11/group).

Parameters	Control	*I. carnea AF* (g/kg body weight)		
		1.0	3.0	7.0
Food consumption	256.1 ± 8.1	252.6 ± 8.5	246.0 ± 9.5	245.4 ± 12.4
Water consumption	546.0 ± 13.6	531.8 ± 30.6	553.2 ± 31.5	549.8 ± 42.8
Maternal body weight gain (g)	29.0 ± 4.42	27.2 ± 2.76	25.8 ± 2.45	8.8 ± 2.48[a]
Maternal body weight at the 20th day of gestation (g)	300.6 ± 8.6	296.9 ± 7.6	303.4 ± 5.2	283.0 ± 7.5
Gravid uterus weight (g)	60.3 ± 3.0	58.8 ± 2.7	57.8 ± 4.7	64.5 ± 2.6
Maternal body weight minus gravid uterus weight (g)	240.3 ± 7.3	238.0 ± 5.9	239.2 ± 3.9	220.4 ± 5.9
Number of viable fetuses per litter	10.4 ± 0.47	10.6 ± 0.4	11.4 ± 0.5	11.6 ± 0.5
Placental weight (g)	0.44 ± 0.01	0.45 ± 0.01	0.44 ± 0.01	0.44 ± 0.02
Fetal weight (g)	3.5 ± 0.08	3.51 ± 0.06	3.57 ± 0.07	3.46 ± 0.1
Fetal length (cm)	3.66 ± 0.01	3.63 ± 0.02	3.62 ± 0.02	3.56 ± 0.03
Preimplantation loss (%)	5.1 ± 4.2	4.8 ± 3.2	5.3 ± 2.7	5.2 ± 3.4
Postimplantation loss (%)	7.1 ± 3.3	8.1 ± 2.9	6.6 ± 3.7	8.3 ± 3.4

[a] $p \leq 0.05$ compared with controls.

within the reference range for the species and may be considered physiologically normal (data not shown). No fetal skeletal or visceral malformations associated with *I. carnea* AF ingestion were detected (Table 2, $p > 0.05$).

Dams from the IC7 group showed diffuse cytoplasmic vacuolization in the liver and kidney when compared with control dams (data not shown). Similarly, the evaluation of the placental tissue from dams that received the highest dose showed thickening of the labyrinth zone and a decrease in the thickness of the junctional zone, which resulted in a 4% reduction in the total junctional area (control group 17.4% and IC7 group 13.04%; $p > 0.05$). Lectin histochemistry evaluation revealed that treatment with *I. carnea* promoted different lectin binding patterns in different areas of placental tissue. Thus, in the junctional zone, there was an accumulation of carbohydrates linked to PNA, WGA, sWGA and DBA; in the labyrinth zone, there was an accumulation of carbohydrates linked to WGA and sWGA; and in the chorionic plate, there was an accumulation of carbohydrates linked to PNA and DBA (Table 3). The percentage of apoptotic cells in the placental tissue was unaffected by the treatments ($p > 0.05$), with 73.5% in the control group and 76% in the IC7 group.

3.3. Postnatal evaluation

The gestation length and litter size of the dams that received different doses of *I. carnea* AF from the 6th to the 19th day of gestation were unaffected by the treatment (Table 4, $p > 0.05$). A lower litter weight was observed in the group that received the highest dose of *I. carnea* (IC7 group) compared to the control group (Table 4, $p < 0.05$). Similarly, (Table 4, $p < 0.05$). Reversible hyperflexion of the carpal joints was observed in some pups from IC7. No treatment effect on the body weight of male and female pups at 7, 14 and 21 days after birth was observed (Table 5, $p > 0.05$). Physical milestones were unaffected by the treatments ($p > 0.05$; data not shown). Female pups from all experimental groups exhibited a delay in achieving a negative geotaxis response compared to females from the control group with no dose-response relationship (Table 5, $p < 0.05$).

The histopathological evaluation revealed slight vacuolar degeneration in the hepatic and renal tissues of pups whose mothers received 3 and 7 g/kg *I. carnea* AF from the 6th to the 19th day of gestation (Fig. 1).

4. Discussion

It is well known that one of the significant effects produced by *I. carnea* toxicity, independent of the animal species considered, is the decrease in body weight (Schumaher-Henrique et al., 2003; Hueza et al., 2007; Antoniassi et al., 2007). In fact, in the present study, we verified that although no differences were detected in food consumption between mothers of the four different groups evaluated, it was observed that dams treated with the highest dose of the plant had a much lower body weight gain when compared with the other groups.

Pritchard et al. (1990) suggest that weight loss is caused by neurological changes produced by swainsonine, which would result in anorexia of neural origin. However, no vacuolar lesions were observed in the nervous tissue of dams treated with different doses of AF. Indeed, in all studies performed in our laboratory with *I. carnea*, using rats as an animal model, it has been consistently shown that contrary to goats (De Balogh et al., 1999; Gotardo et al., 2012), ewes (Armién et al., 2011) and bovines (Antoniassi et al., 2007), the intoxication of *I. carnea* in this animal species did not produce any lesions in the CNS (Hueza et al., 2003, 2005, 2007; Schwarz et al., 2003). This assumption is also based on the results obtained by Huxtable and Dorling (1985), who observed that rats treated with high doses of swainsonine (approximately 15 mg/day) for 100–200 days developed neuronal mannosidase storage disease only in areas of the brain not protected by the blood/brain barrier.

Considering that in the present study, no differences were detected between all groups in relation to food consumption of the females during the entire period of gestation, a possibility to explain the decrease in the weight gain of the dams treated with the higher dose of *I. carnea* would be related to the inhibitory action of the plant alkaloids on some sucrases present in the brush border of the small intestine cells, such as the enzymes α-glycosidases and α- and β-galactosidases. Thus, this inhibition would result in less carbohydrate absorption and, consequently, less weight gain (Pan et al., 1993; Asano, 2000). Hence, to better clarify this conjecture, future studies will be performed measuring the sucrase activity as well as the products of its hydrolysis (i.e., fructose and glucose) and the level of sucrose in animals treated with different doses of swainsonine.

It is fairly well known that adverse pregnancy outcomes are strongly linked to disorders of the placenta since fetal growth and development are dependent on nutrient availability, which in turn is related to the

Table 2
Frequency of fetal anomalies in pregnant rats treated by gavage with 7.0 g/kg body weight of *I. carnea* aqueous fraction (AF) from days 6–19 of gestation (n = 11/group).

Parameters	Control	*I.carnea*
Total number of litters	11	11
Total fetuses examined	44	44
Skeletal malformations	0	0
Skeletal anomalies		
Wavy rib	2(4.5%)	2(4.5%)
Bipartite sternebrae	0(0.0%)	1(2.3%)
Incomplete ossification of sternebra	7(12.5%)	7(16.6%)
Abnormally shaped sternebra	7(15.9%)	8 (18.2%)
Absence of organs	0	0
Visceral malformations	0	0
Visceral anomalies		
Peritoneal hemorrhage	3 (6.8%)	2 (4.5%)
Sinuous ureter	1 (2.3%)	2 (4.5%)

Table 3
Lectin binding pattern in different areas of placental tissue of pregnant rats treated by gavage with 7.0 g/kg body weight of *I. carnea* aqueous fraction (AF) from days 6–19 of gestation (n = 11/group).

Lectin	Junctional zone		Labyrinth zone		Chorionic plate	
	Control	*I.carnea*	Control	*I.carnea*	Control	*I.carnea*
PNA	-	++	-	-	++	+++
sWGA	-	+	-	++	-	-
WGA	+	+++	+	++	++	++
DBA	-	+	-	-	++	+++
RCA-1	++	++	+	+	++	++
BS-1	+++	+++	+	+	+	+

Table 4
Data relating to births of rats treated by gavage with different doses of *I. carnea* aqueous fraction (AF) from days 6–19 of gestation (n = 10/group) and offspring measurements at postnatal day one (mean ± S.E.M.).

Parameters	Control	*I. carnea AF* (g/kg body weight)		
		1.0	3.0	7.0
Gestation length (days)	22.8 ± 0.1	22.7 ± 0.1	22.7 ± 0.1	22.9 ± 0.1
Number of pups per litter	10.3 ± 0.6	11.6 ± 0.4	10.2 ± 0.5	10.1 ± 0.6
Number of male pups	4.9 ± 0,7	6.4 ± 0.4	5.1 ± 0.4	4.1 ± 0.5
Number of female pups	5.4 ± 0.8	5.0 ± 0.4	5.1 ± 0.5	6.0 ± 0.7
Litter weight (g)	69.3 ± 2.8	72.1 ± 2.6	62.8 ± 3.9	59.6 ± 3.3[a]
Weight of male pups (g)	7.2 ± 0.2	6.6 ± 0.2	6.5 ± 0.2	6.2 ± 0.2[a]
Weight of female pups (g)	6.6 ± 0.1	6.1 ± 0.2	6.1 ± 0.2	5.6 ± 0.2[a]
Weight litter after culled (g)	53.5 ± 1.2	49.6 ± 1.6	50.1 ± 2.0	47.4 ± 1.4[a]

[a] $p \leq 0.05$ compared with controls.

Table 5
Body weight (BW) at postnatal days (PND) 7, 14 and 21 (g), neuromotor and reflex development milestones (day) of male and female offspring rats from dams treated by gavage with different doses of *I. carnea* aqueous fraction (AF) from days 6–19 of gestation (n = 40/group/sex; mean ± S.E.M.).

Parameters		Control	*I. carnea AF* (g/kg body weight)		
			1.0	3.0	7.0
BW PND7	♂ (40)[a]	17.4 ± 0.7	16.4 ± 0.3	16.4 ± 0.4	15.6 ± 0.5
	♀ (40)	15.8 ± 0.4	16.0 ± 0.5	15.4 ± 0.4	14.5 ± 0.5
BW PND14	♂ (35)	35.3 ± 1,0	32.8 ± 0.9	33.3 ± 0.9	32.1 ± 0.6
	♀ (35)	32.1 ± 0.5	31.8 ± 1.1	31.6 ± 0.8	30.6 ± 0.5
BW PND21	♂ (30)	55.7 ± 1.4	51.6 ± 1.0	52.6 ± 1.3	51.8 ± 1.3
	♀ (30)	51.1 ± 0.9	49.9 ± 1.4	49.3 ± 1.2	48.1 ± 0.9
Adult gait	♂ (10)	13.2 ± 0.3	13.4 ± 0.1	13.6 ± 0.2	13.6 ± 0.3
	♀ (10)	13.2 ± 0.3	13.6 ± 0.1	13.5 ± 0.2	13.5 ± 0.2
Surface righting reflex	♂ (10)	4.22 ± 0.1	4.25 ± 0.1	4.18 ± 0.1	4.37 ± 0.1
	♀ (10)	4.55 ± 0.2	4.41 ± 0.3	4.18 ± 0.1	4.75 ± 0.2
Negative geotaxis	♂ (10)	7.44 ± 0.6	9.08 ± 0.5	9.09 ± 0.6	9.37 ± 0.5
	♀ (10)	6.89 ± 0.6	9.33 ± 0.5*	10.0 ± 0.5*	9.12 ± 0.7*
Startle reflex	♂ (10)	12.3 ± 0.2	12.2 ± 0.2	12.3 ± 0.2	12.2 ± 0.2
	♀ (10)	12.2 ± 0.1	12.3 ± 0.1	12.2 ± 0.2	12.2 ± 0.2

*$p \leq 0.05$ compared with controls.
[a] Number of observations.

maternal diet, uteroplacental blood supply, placental villous development, and the capacity of the villous trophoblast and fetoplacental circulation to transport these nutrients (Sarkar et al., 2014; Burton and Jauniaux, 2018, Elkin et al., 2020). Thus, it should be supposed that the chemical-induced histopathological changes in the placenta of rats are an essential part of a safety evaluation to understand the mechanism of teratogenicity and developmental toxicity; however, in general, morphological or histopathological assessment of placental development and abnormalities is rare and incomplete in experimental studies.

In the present study, when analyzing the placental tissue of those females treated with the highest dose of the plant under light microscopy, it was possible to verify that there was thickening in the labyrinth zone, with an apparent narrowing of the sinusoids. The labyrinth zone consists of fetal and maternal blood spaces separated by vascular endothelium and specialized trophoblast cell types, and it is located at the fetal interface and corresponds to the area of maternal-fetal exchanges; thus, labyrinth zone dysfunction is known to cause unfavorable impacts on embryonic development (Enders and Schlafke, 1969; Soares et al., 1987; Ali et al., 2018). In this manner, the narrowness of the maternal sinusoids in the labyrinth zone observed in the present study may be indicative of a decrease in placental blood circulation, resulting in impairment of those exchanges of substances between mother and fetus. In fact, the decrease in the supply of oxygen or substrates to the fetus, in general, due to changes in uteroplacental blood flow, is often associated with intrauterine growth restriction (Burton and Jauniaux, 2018).

The analysis of the placental junctional zone performed in this research showed a significant decrease in its thickness in females submitted to the treatment with the highest dose (7.0 g/kg) of *I. carnea*. The junctional zone separates the labyrinth from the maternal decidua and consists of spongiotrophoblasts, glycogen trophoblasts and a discontinuous layer of trophoblast giant cells (TGCs) with large polyploid nuclei. Taking into account that it is well known that apoptosis is essential for the normal development, remodeling, and aging of the placenta (Austgulen et al., 2002), we hypothesize that swainsonine and/or other active ingredients could interfere with the apoptosis process in the cells of the

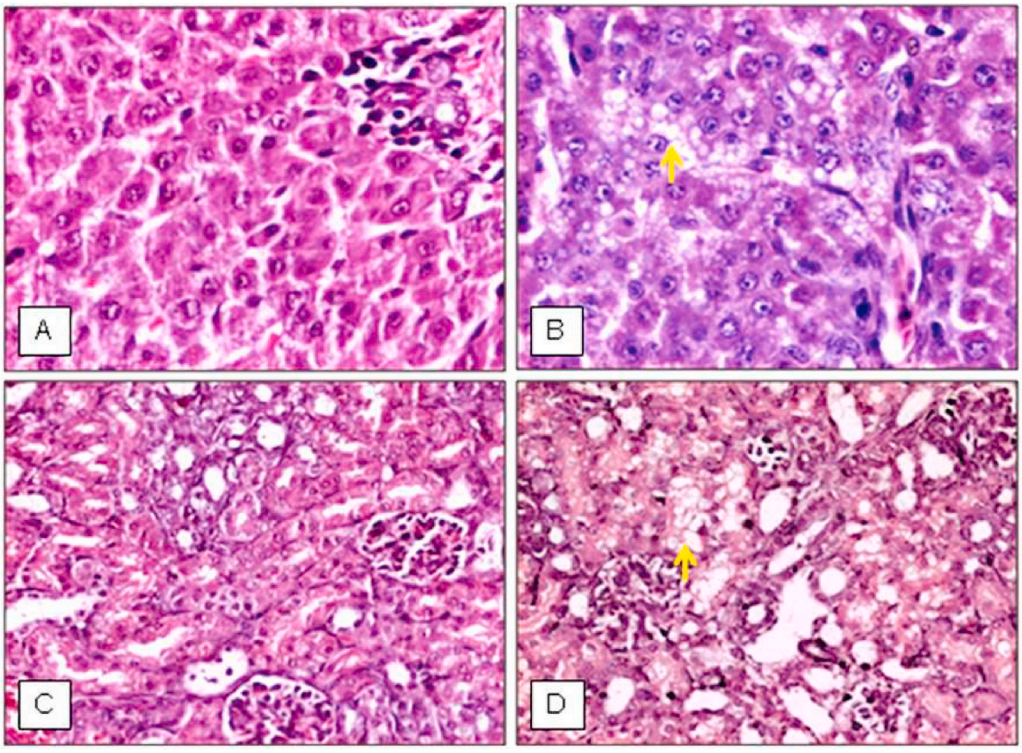

Fig. 1. Light photomicrography of the liver (40x) and kidney (20x) from pups on postnatal day seven: (A) Liver from control pup. (B) Liver from pup whose mother received 7 g/kg body weight of *I. carnea* dry leaves, as the aqueous fraction, during gestation, showing hepatocyte vacuolar degeneration (arrow). (C) Kidney from control pup. (D) Kidney from pup whose mother received 7 g/kg body weight of *I. carnea* dry leaves, as the aqueous fraction, during gestation, showing vacuolar degeneration in the cortical tubular epithelium.

junctional layer, leading to placental dysfunction. However, TUNEL, an assay that relies on the detection of DNA strand breaks in situ by labeling them with fluorochromes, thus identifying and quantifying apoptotic cells, did not reveal any differences in this layer between control and experimental animals. Hence, studies employing a more specific immunohistochemical technique, that is, the method of caspase-3, an apoptotic marker, in the placenta will be carried out to better evaluate this assumption.

Although changes in the placental tissue were detected and visualized by light microscopy, no vacuolar lesion, which is the hallmark of poisoning by *I. carnea*, was observed; thus, it became essential to use a specific diagnostic method for the detection of sugar residues, which could be stored in the cell but not evidenced by the HE technique. For this task, the lectin-histochemistry methodology was used. In fact, it was possible to verify several changes in the placenta of females from the group treated with the highest dose of the plant. In the junctional zone, specifically in the giant trophoblastic cells and in the chorionic plate, there was an accumulation of galactose-β-(1–3)-N-acetylgalactosamine (marked by PNA), which is indicative of the inhibitory action of calystegines B1 and B2 on the α- and β-galactosidase enzymes. The accumulation of this same sugar was also found in the research conducted by Cholich et al. (2009) in pancreatic cells of guinea pigs treated with *I. carnea* for 45 days. Other studies in adult goats (Armién et al., 2007) and pregnant sheep (Armién et al., 2011) also showed PNA-positive cells in perivascular cells of the brain and in the renal tubular epithelium of animals intoxicated with *I. carnea*.

The analysis of the placental tissue by lectin DBA revealed an alteration markedly similar to that observed by staining with PNA. Thus, the females that received 7.0 g/kg of the plant stained positively for this lectin in the same areas of the placenta (i.e., in giant trophoblastic cells and in the chorionic plate). It is known that DBA has an affinity for the carbohydrate α-D-N-acetyl-galactosamine; therefore, we can suggest the inhibitory action of calystegines B1 and B2 on the enzymes α- and β-galactosidases.

Immunohistochemistry based on the WGA and s-WGA lectin bonds in oligosaccharides is commonly used to detect these nonmetabolized sugars, rich in β-N-acetylglycosamine and N-acetyl-neuroamino acid residues, which accumulate inside lysosomes, leading to the process of lysosomal vacuolar degeneration and altering this characteristic of intoxication produced by swainsonine. In the present study, when WGA and s-WGA labeling was used, it was possible to observe a higher concentration of oligosaccharides in the placental tissue of dams treated with the highest dose of *I. carnea* extract, with this positive marking evidenced in spongiotrophoblast cells and inside giant trophoblastic cells (junctional zone), as well as on the surface of the labyrinthine space (marked positive staining for WGA) and in the junctional zone and trophoblasts in the region in contact with maternal blood in the labyrinth (marking s-WGA). This finding clearly indicates the expression of β-D-N-acetylglucosamine and acetylneuraminic acid, the main carbohydrates accumulated in this toxicosis. The staining for WGA and sWGA lectins observed here has been described in the neurons of goats (Armién et al., 2007), guinea pigs (Cholich et al., 2009) and ewes (Armién et al., 2011) with *I. carnea* intoxication.

One of the most important limitations of the protocols that have been used to assess developmental toxicity for potential exposure risks of numerous chemical substances during fetal development is the lack of examination of the offspring during the postnatal period, which could lead to mistakes in the assessment of the developmental toxic effects of the substances under examination (Claudio et al., 2000). For example, heptachlor, an organochlorine insecticide, can cause increased postnatal death only after PND6. In addition, this substance can also cause the formation of cataracts; however, this effect is manifested 21 days after birth (Mestitzova, 1967). In the same manner, many studies in our laboratory have shown that administration of toxicants (Soto-Blanco and Górniak, 2004; Sousa et al., 2007; Hueza et al., 2007; Gotardo et al., 2011, 2015, 2016, 2019) or feed restriction (Ricci et al., 2014; Gotardo et al., 2017) to mothers during organogenesis produces minimal or no alteration of the pups when evaluated using conventional protocols (i.e., assessing developmental effects in the fetus just before term); however, the deleterious effects in the offspring manifested belatedly.

In fact, again, we verified here that among all the reproductive toxicity parameters analyzed in the conventional protocol, none showed significant differences between the control and experimental groups. Similarly, no alterations were found in either the visceral analysis or in the skeletal morphology of the fetuses. On the other hand, when evaluating the reproductive performance of females treated during the same period of pregnancy but allowed to give birth naturally, a decreased weight of male and female pups on PND1 was verified with the highest dose of AF *I. carnea*.

Moreover, the neurobehavioral study of pups conducted in this research also showed changes. Thus, we used this model to reflect the negative geotaxis of pups, which depicts the degree of maturation, especially of the vestibular system, which is involved in the spatial relationship of the animals, and motor development (Patin et al., 2004). In this study, it was observed that exposure of pregnant females to the highest dose of AF *I. carnea* caused a delay in the onset of this parameter in female offspring, suggesting a delay in the maturation of nervous structures involved in motor skills, particularly the cerebellum. At this point, little could be elucidated regarding why this test does not change in male pups; however, it may suggest that the action of testosterone on male offspring, which starts in the last days of gestation and extends to close to 10 days of postnatal life, produces neurochemical actions (Fernandes et al., 1999) that protect the male offspring against the possible effects of the AF plant in the vestibular system, favoring normal maturation. These same data were also reported by Schwarz et al. (2003), who studied the effects of the extract of *I. carnea* in pregnant rats using double the dose used here (the levels of the swainsonine and the nortropane alkaloids were approximately the same as the AF *I. carnea* used in this study).

The external changes observed on day 1 postpartum in this study, including hyperflexion of the carpal joints, were observed in pups from females treated with AF *I. carnea*; however, over time, there was regression of this alteration. These findings were also observed by other authors in different animal species when studying the effects of perinatal locoweeds in sheep (James, 1972; Keeler, 1988), as well as in goats and rats treated with *I. carnea* during pregnancy (Schwarz et al., 2003; Gotardo et al., 2012). In the same manner, the histopathological study of both mothers and pups revealed that the liver and kidneys presented the characteristic vacuolar degeneration produced by the indolizidine alkaloids. Alterations in these organs were also detected in pups on PND15 and PND22. These data support the transplacental passage of swainsonine, as reported in previous studies performed in this laboratory in rats (Hueza et al., 2007) and goats (Gotardo et al., 2012).

5. Conclusion

In conclusion, the results from this study show that the toxic principle of *I. carnea* produces injury in the placenta, and for the first time, the typical vacuolization produced by swainsonine in this organ was characterized. It was also possible to confirm earlier works performed in rats (Hueza et al., 2007; Schwarz et al., 2007), ewes (Armién et al., 2011) and goats (Gotardo et al., 2012) showing that intoxication during gestation can cause toxic effects *in utero* in mothers and fetuses, but these deleterious effects are better demonstrated using postnatal evaluation. However, considering that rats prove to not be a good model for the reproduction of neuronal storage disease, future studies will be conducted, using goats as animal model, to better characterize the role of the placentotoxic effect of the plant in the fetal injury.

Ethical statement

This study was conducted at the University of São Paulo (USP). The

procedures were approved by the USP Animal Ethics Committee (protocol number 1091/2007), and all animal care and handling were performed by experienced personnel under veterinary supervision.

Funding

This work is part of Master's thesis presented by Luciana Lucinio Lippi to the Department of Pathology, School of Veterinary Medicine and Animal Sciences, University of São Paulo, Brazil, supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP Proc No. 06/58728-6).

Author contributions

L.L.L., and S.L.G. conceived and designed the experiments; L.L.L. performed the experiments; L.L.L., E.M.A.F.B. and K.B.V. analyzed the samples; A.T.G. and L.L.L. analyzed the data; A.T.G., L.L.L. and S.L.G. wrote the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Ali, H., Ghareeb, A.E.W.E., Taha, E.L.S.F., Hamdi, H., 2018. Placental toxicity in rats after administration of broncholytic drug (Mucophylline) A morphological, histological, histochemical and immunohistochemical studies. J. Clin. Exp. Tox. 2 (1), 1–9.

Antoniassi, N.A.B., Ferreira, E.V., dos Santos, C.E.P., de Arruda, L.P., Campos, J.L.E., Nakazato, L., Colodel, E.M., 2007. Intoxicação espontânea por *Ipomoea carnea* subsp. fistulosa (Convolvulaceae) em bovinos no Pantanal Matogrossense. Pesqui. Vet. Bras. 27 (10), 415–418.

Armién, A.G., Tokarnia, C.H., Peixoto, P.V., Frese, K., 2007. Spontaneous and experimental glycoprotein storage disease of goats induced by *Ipomoea carnea* subsp fistulosa (Convolvulaceae). Vet. Pathol. 44, 170–184.

Armién, A.G., Tokarnia, C.H., Peixoto, P.V., Barbosa, J.D., Frese, K., 2011. Clinical and morphologic changes in ewes and fetuses poisoned by *Ipomoea carnea* subspecies. J. Vet. Diagn. Invest. 23, 221–232.

Asano, N., 2000. Water soluble nortropane alkaloids in crude drugs, edible fruits and vegetables: biological activities and therapeutic applications. Mech. Ageing Dev. 116, 155–156.

Austgulen, R., Chedwick, L., Vogt, I.C., Vatten, L., Craven, C., 2002. Trophoblast apoptosis in human placenta at term as detected by expression of a cytokeratin 18 degradation product of caspase. Arch. Pathol. Lab Med. 126, 1480–1486.

Austin, D.F., Huáman, Z., 1996. A synopsis of *Ipomoea* (convolvulaceae) in the americas. Taxon 45, 3–38.

Burton, G.J., Jauniaux, E., 2018. Pathophysiology of placental-derived fetal growth restriction. Am. J. Obstet. Gynecol. 218 (2S), S745–S761.

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.

Claudio, L., Kwa, W., Russell, A., Wallinga, D., 2000. Testing methods for developmental neurotoxicity of environmental chemicals. Toxicol. Appl. Pharmacol. 164, 1–14.

Damir, A.H., Adam, S.E.I., Tartour, G., 1987. The effects of *Ipomoea carnea* on goats and sheep. Vet. Hum. Toxicol. 29 (4), 316–319.

De Balogh, K.I.M., Dimande, A.P., Van Der Lugt, J.J., Molyneux, R.J., Naudé, T.W., Welman, W.G., 1999. A lysossomal storage disease induced by *Ipomoea carnea* in goats in Mozambique. J. Vet. Diagn. Invest. 11, 266–273.

Elkin, E.R., Harris, S.M., Su, A.L., Lash, L.H., Loch-Caruso, R., 2020. Placenta as a target of trichloroethylene toxicity. Environ. Sci. Process. Impacts. 22 (3), 472–486.

Enders, A.C., Schlafke, S., 1969. Cytological aspects of trophoblast-uterine interaction in early implantation. Am. J. Anat. 125, 1–29.

Fernandes, C., Gonzáles, M.I., Wilson, C.A., File, S.E., 1999. Factor analysis shows that female rat behaviour is characterized primarily by activity, male rats are driven by sex and anxiety. Pharmacol., Biochem. Behav. 64, 731–738.

Gardner, D.R., Molyneux, R.J., Ralphs, M.H., 2001. Analysis of swainsonine: extraction methods, detection, and measurement in populations of locoweeds (Oxytropis spp.). J. Agric. Food Chem. 49, 4573–4580.

Gotardo, A.T., Pfister, J.A., Barbosa-Ferreira, M., Górniak, S.L., 2011. Effects of prepartum ingestion of *Ipomoea carnea* on postpartum maternal and neonate behavior in goats. Birth Defects Res. B Dev. Reprod. Toxicol. 92, 131–138.

Gotardo, A.T., Schumaher-Henrique, B., Pfister, J.A., Traldi, A.S., Maiorka, P.C., Spinosa, H.S., Górniak, S.L., 2012. The use of ultrasonography to study teratogenicity in ruminants: evaluation of *Ipomoea carnea* in goats. Birth Defects Res. B Dev. Reprod. Toxicol. 95, 289–295.

Gotardo, A.T., Pavanelli, E.L., Carvalho, H.F., Lemes, K.M., Arruda, R.P., Kempinas, W. G., Górniak, S.L., 2014. Endocrine disrupter action in ruminants: a study of the effects of Ipomoea carnea in adult male goats. Small Rumin. Res. 119, 81–87.

Gotardo, A.T., Hueza, I.M., Manzano, H., Maruo, V.M., Maiorka, P.C., Górniak, S.L., 2015. Intoxication by cyanide in pregnant sows: prenatal and postnatal evaluation. J. Toxicol. 2015, 1–9.

Gotardo, A.T., Pfister, J.A., Raspantini, P.C.F., Górniak, S.L., 2016. Maternal ingestion of Ipomoea carnea: effects on goat-kid bonding and behavior. Toxins 8, 74.

Gotardo, A.T., Dipe, V.V., Hueza, I.M., Górniak, S.L., 2017. Maternal feed restriction during pregnancy in Wistar rats: evaluation of offspring using classical and immunoteratology protocols. Hum. Exp. Toxicol. 36, 603–615.

Gotardo, A.T., Mattos, M.I.S., Hueza, I.M., Górniak, S.L., 2019. The effect of Cynara scolymus (artichoke) on maternal reproductive outcomes and fetal development in rats. Regul. Toxicol. Pharmacol. 102, 74–78.

Haraguchi, M., Gorniak, S.L., Ikeda, K., Minami, Y., Kato, A., Watsom, A.A., Nash, R.J., Molyneux, R.J., Asano, N., 2003. Alkaloidal components in the poisonous plant, *Ipomoea carnea* (Convolvulaceae). J. Agric. Food Chem. 51, 4995–5000.

Hueza, I.M., Dagli, M.L.Z., Górniak, S.L., 2003. Toxic effects of prenatal *Ipomoea carnea* administration to rats. Vet. Hum. Toxicol. 45, 298–302.

Hueza, I.M., Guerra, J.L., Haraguchi, M., Asano, N., Górniak, S.L., 2005. The role of alkaloids in *Ipomoea carnea* toxicosis: a study in rats. Exp. Toxicol. Pathol. 57, 53–58.

Hueza, I.M., Guerra, J.L., Haraguchi, M., Gardner, D.R., Asano, N., Ikeda, K., Górniak, S. L., 2007. Assessment of the perinatal effects of maternal ingestion of *Ipomoea carnea* in rats. Exp. Toxicol. Pathol. 58, 439–446.

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.

Ilekis, J.V., Tsilou, E., Fisher, S., Abrahams, V.M., Soares, M.J., Cross, J.C., Zamudio, S., Illsley, N.P., Myatt, L., Colvis, C., Costantine, M.M., Haas, D.M., Sadovsky, Y., Weiner, C., Rytting, E., Bidwell, G., 2016. Placental origins of adverse pregnancy outcomes: potential molecular targets- an executive workshop summary of the eunice kennedy shriver national institute of child health and human development. Am. J. Obstet. Gynecol. 215, 1–46.

James, L.F., 1972. Syndromes of locoweed poisoning in livestock. Clin. Toxicol. 5, 567–573.

Keeler, R.F., 1988. Livestock models of human birth defects, reviewed in relation to poisonous plants. J. Anim. Sci. (Sofia) 66, 2414–2427.

Mestitzova, M., 1967. On reproduction studies on the occurrence of cataracts in rats after long-term feeding of the insecticide heptachlor. Experientia 23, 42–43.

Molyneux, R.J., McKenzie, R.A., O'Sullivan, B.M., Elbein, A.D., 1995. Identification of the glycosidase inhibitors swainsonine and calystegine B2 in Weir vine (Ipomoea sp. Q6 [aff. calobra]) and correlation with toxicity. J. Nat. Prod. 58 (6), 878–886.

Moremen, K.W., Robbins, P.W., 1991. Isolation, characterization, and expression of cDNAs encoding murine alpha-mannosidase II, a Golgi enzyme that controls conversion of high mannose to complex N-glycans. J. Cell Biol. 115 (6), 1521–1534.

Pan, Y.T., Ghidoni, J., Elbein, A.D., 1993. The effects of castanospermine and swainsonine on the activity and synthesis of intestinal sucrase. Arch. Biochem. Biophys. 303, 134–144.

Patin, V., Vincent, A., Lordi, B., Caston, J., 2004. Does prenatal stress affect the motoric development of rat pups? Dev. Brain Res. 149, 85–92.

Pfister, J.A., Astorga, J.B., Panter, B.L., Stegelmeier, R.J., Molyneux, R.J., 2006. Maternal ingestion of locoweed I. Effects on Ewe-lamb bonding and behavior. Small Rumin. Res. 65, 51–53.

Pritchard, D.H., Huxtable, C.R., Dorling, P.R., 1990. Swainsonine toxicosis supreess appetite and retards growth in weanling rats. Res. Vet. Sci. 46, 228–230.

Ricci, E.L., Bernardi, M.M., Górniak, S.L., Spinosa, H.S., 2014. Behavioral teratogenicity induced by maternal food restriction: maternal cannibalism and poor reflex development in offspring. Biotemas 27, 185.

Roescher, A.M., Timmer, A., Erwich, J.J.H.M., Bos, A.F., 2014. Placental Pathology, perinatal death, neonatal outcome, and neurological development: a systematic review. PloS One 9 (2), e89419.

Sarkar, A.A., Nuwayhid, S.J., Maynard, T., Ghandchi, F., Hill, J.T., Lamantia, A.S., Zohn, I.E., 2014. Hectd1 is required for development of the junctional zone of the placenta. Dev. Biol. 392, 368–380.

Schumaher-Henrique, B., Górniak, S.L., Dagli, M.L.Z., Spinosa, H.S., 2003. Toxicity of long term administration of *Ipomoea carnea* to growing goats: clinical, biochemical, haematological and pathological alterations. Vet. Res. Commun. 27, 311–319.

Schwarz, A., Hosomi, R.Z., Flório, J.C., Bernardi, M.M., Górniak, S.L., Spinosa, H.S., 2007. Rats offspring exposed to Ipomoea carnea and handling during gestation: neurochemical evaluation. Braz. Arch. Biol. Technol. 50, 425–433.

Schwarz, A., Górniak, S.L., Bernardi, M.M., Dagli, M.L.Z., 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 (5), 615–626.

Soares, M.J., Schaberg, K.D., Pinal, C.S., De, S.K., Bhatia, P., Andrews, J.K., 1987. Establishment of a rat placental cell line expressing characteristics of extraembryonic membranes. Dev. Biol. 124, 134–144.

Soto-Blanco, B., Górniak, S.L., 2004. Prenatal toxicity of cyanide in goats - a model for teratological studies in ruminants. Theriogenology 62, 1012–1026.

Sousa, A.B., Maiorka, P.C., Gonçalves, I.D., de Sá, L.R.M., Górniak, S.L., 2007. Evaluation of effects of prenatal exposure to the cyanide and thiocyanate inwistar rats. Reprod. Toxicol. 23, 568–577.

Staples, R., Schnell, V.L., 1964. Refinements in rapid clearing technic in the KOH-Alizarin Red S method for fetal bone. Stain Technol. 39, 61–63.

Tokarnia, C.H., Dobereiner, J., Peixoto, P.V., 2000. Plantas Toxicas Do Brasil, second ed. Helianthus, Rio de Janeiro.

Tulsiani, D.R.P., Touser, O., 1983. Swainsonine inhibits the biosynthesis of complex glycoproteins by inhibition of Golgi manosidase II. J. Biol. Chem. 224, 594–600.
Wilson, J.C., 1965. Methods for administering agents and detecting malformation in experimental animal. In: Wilson, J.C., Warkany, J. (Eds.), Teratology: Principles and Techniques. University of Chicago Press, Chicago, pp. 262–327.
Wise, L.D., Beck, S.L., Beltrame, D., Beyer, B.K., Chahoud, I., Clark, R.L., Clark, R., Druga, A.M., Feuston, M.H., Guittin, P., Henwood, S.M., Kimmel, C.A., Lindstrom, P., Plalmer, A.K., Petrere, J.A., Solomon, H.M., Yasuda, M., York, R.G., 1997. Terminology of developmental abnormalities in common laboratory mammals (version 1). Teratology 55, 249–292.