Embryotoxic effects of prenatal treatment with *Ipomoea carnea* aqueous fraction in rats

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Abstract

The embryotoxic effects of prenatal daily exposure to 0.0, 0.7, 3.0 or 15.0 mg/kg of the aqueous extract (AQE) from *Ipomoea carnea (I. carnea)* dried leaves on gestational days 5–21 were studied in rats. Maternal reproductive performance, skeletal and visceral abnormalities, and malformations were evaluated. Moreover, anatomopathological findings in dams following the treatment were recorded. Regarding the dams, our results show that body weight, weight gain, food and water consumption, and reproductive performance were all unaffected by exposure to the different doses of the AQE. Nonetheless, dams treated with AQE presented a dose-dependent cytoplasmic vacuolation in the liver, kidneys, thyroid and adrenal glands. Fetal examination did not show external abnormalities or malformations. Evidences of several skeletal and visceral abnormalities were found, particularly after the higher dose of AQE. A reduced ossification centers were also detected. The present data show that prenatal ingestion of the *I. carnea* AQE in rats induces embryotoxicity. These effects are attributed to an active principle from *I. carnea* acting on maternal homeostasis, or directly in the conception.

Key words:
Ipomoea.
Teratogens.
Prenatal care.

Introduction

I. carnea is a toxic plant widely distributed in Brazil[1] and other tropical countries[2]. Two kinds of toxic principles were isolated from the plant, the nortropane alkaloids calystegines B_1, B_2, B_3 and C_1 and mainly the indolizidine alkaloid swainsonine.[3,4] The latter alkaloid has a known toxic mechanism of action, being a potent inhibitor of two distinct intracellular enzymes, the acidic or lysosomal *á*-mannosidase and the Golgi mannosidase II. However, with respect to calystegines B_2 and C_1, these nortropane alkaloids are only recognized as inhibitors of α-galactosidase and β-glucosidase enzymes, respectively.[5] Recent studies conducted in our lab, comparing the histological effects of each calystegine, in the same concentration contained in *I. carnea* aqueous fraction (AQE) administered to rats, did not show the characteristic vacuolar lesions observed in animals treated solely with swainsonine.[6] Thus, whether all these alkaloids together enhance the effects produced by swainsonine intoxication on *I. carnea* is unknown.[7]

I. carnea promotes in livestock a toxicosis histologically characterized by vacuolated cells in different organs. The toxic principles of *I. carnea* are the alkaloids swainsonine and calystegines B1, B2, B3 and Cl. However, it has not been determined whether the effects observed in rats treated with this plant are only due to swainsonine or if the calystegines have some additive toxic effect. The histopathologic study showed that while calystegines did not produce any toxic effects, swainsonine and AQE promoted vacuolation in different organs, being more severe in the animals from the *I. carnea* AQE group and extensible to other organs evaluated, suggesting. that calystegines may act as coadjuvants of

swainsonine in *I. carnea* toxicosis. [6]

It has long been reported that long-term ingestion of *I. carnea* (a tropical plant of the convolvulaceae family) leads to neurobehavioral effects in goats, cattle and sheep.[8] However, there are only a few reports about its effects on the offspring if grazed by pregnant animals. Schwarz et al. [9] described several changes on developmental parameters induced by AQE prenatal exposure this extract. No changes in behavior or neurochemical parameters in adulthood were observed, suggesting that, in rats, these toxic effects are reversible.

The main objective of this investigation was to study possible embryotoxic effects of *I. carnea* AQE on rats exposed during the organogenesis period. We examined the visceral and skeletal development of the offspring from *I. carnea* treated dams. Furthermore, we investigated possible anatomopathological changes induced in dams during this treatment.

Material And Method

Plant

I. carnea leaves were collected in May, 2004 from plants cultivated at the Research Center for Veterinary Toxicology (CEPTOX), University of São Paulo (USP), Pirassununga, Brazil.

Preparation of the aqueous fraction extract (AQE)

Fresh leaves were first triturated with ethyl alcohol (97º Gay Lussac) in a blender, macerated for 72 hours in ethyl alcohol (97º Gay Lussac), and filtered using a Büchner funnel. The filtrate was then evaporated under reduced pressure, and the product obtained was reserved. The recovered ethanol was again mixed to the leaf residue for a 24 -hour maceration, following by additional stages of filtration and evaporation under reduced pressure; the resulting product was again reserved. This procedure was repeated twice over, and the four products were pooled, composing the final extract, which was diluted in distilled water and filtered through paper. The filtered portion, ethanolic fraction, was treated with butanolic alcohol and separated with a decantation funnel. This procedure originated the AQE that was stored at -20º C.

Animals

Male and female Wistar rats from the Department of Pathology (School of Veterinary Medicine, University of São Paulo), weighting 180-200 g, and aged approximately 90 days were used. The animals used in this study were maintained in accordance with The Guide for the Care and Use of Laboratory Animal, National Research Council, USA (1996).

Procedures

Treatment with I. carnea AQE, reproductive parameters and maternal data

*Se*xually naive female rats (n = 40) were mated with males previously tested as fertile (two females and one male per cage). Pregnancy was determined by the presence of spermatozoa in vaginal smears on the following morning, designated as gestation day 1 (GD1).[10] Pregnant rats were removed from the male cage, and kept in isolation. On GD5, the dams were divided into four groups (one control and three experimental groups). The experimental groups (n=10 animals/group) were treated orally by gavage, once a day from GD5 to GD 21, with 0.7, 3.0 or 15.0 mg/kg of AQE. The control group received tap water by gavage.

The pregnant rats were weighed on GD1, GD5, GD7, GD11, GD14, GD17 and GD21. Food and water consumption during pregnancy were also assessed.

On GD21, the dams were submitted to euthanasia and their uterine horns were removed. The number of implantations, resorptions, dead and live fetuses, and corpora lutea were recorded. Additionally, liver, kidneys, thyroid and adrenal glands were removed from the dams, weighed and analyzed; tissue specimens were fixed in 10% neutral buffered formaldehyde forhistolopathological

routine processing , embedded in paraffin, and 5-ìm-thick sections were cut and stained with hematoxylin and eosin for light microscopy evaluation.

The fetuses were examined macroscopically for external abnormalities, and individually weighed, as were their placentas. These data were used to calculate litter weight, as well as the placental litter weight. The following parameters were analyzed: skull shape, ears and palate implantation, tail and foot conformation, anal drilling, among others. The percentage of preimplantation loss was calculated as: number of corpora lutea – number of implantations x 100/ number of corpora lutea, and percentage of postimplantation loss was calculated as: number of implantation – number of live fetuses x 100/number of implantations.

Under deep anesthesia, half of each litter was fixed in Bouin's solution for subsequent visceral examination, following serial section as described by Wilson [11], and the other half litter was stained with Alizarin red according to the technique of Staples and Schenell [12] to identify skeletal alterations. The extent of ossification was evaluated using the parameters proposed by Aliverti et al.[13]. Data are presented as the number of fetuses or litters affected, i.e., the total number of fetuses or the number of litters that present abnormalities or malformations.

Statistical analysis

One-way ANOVA was used to compare data from body weight, weight gain, and reproductive performance of dams. Two-way ANOVA was employed to analyze food and water consumption by the dams. Several other parameters were evaluated as frequencies of occurrence and compared by means of the qui^2 Square Test [14]. The GraphPad Instat software package [15] was used throughout this study. In all cases, results were considered to be significant when $p < 0.05$.

Results

Few significant differences in the weight gain of dams treated with the AQE were observed, when compared to the control group (Figure 1). Food and water consumption were not altered by these treatments on any group (data not show).

The reproductive performance of the dams treated during organogenesis was similar to that of control animals (Table 1). There was no significant difference in the number of implantation sites, number of resorptions, number of live fetuses per litter, number of corpora lutea, fetal weight and

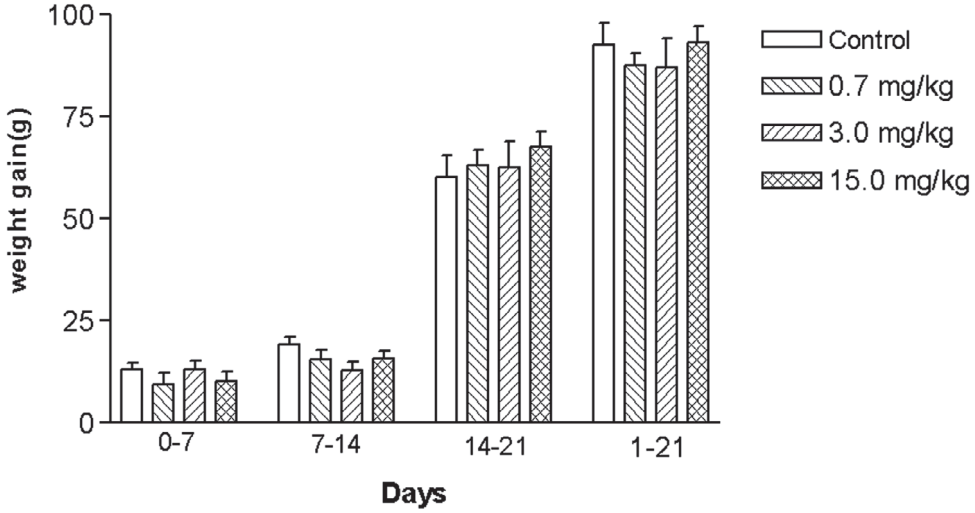

Figure 1 - Weight gain of pregnant rats exposed during pregnancy to 0.7, 3.0 or 15.0 mg/kg of *I. carnea* AQE. Data are presented as means ± S.E.M.

Table 1 - Reproductive performance of rats treated with 0.7, 3.0 or 15 mg/kg of *I. carnea* AQE from GD6 to GD 21 . This table showed the means and the standard error or percentage

Parameters	Control (n = 10)	0.7 mg/kg/day (n = 10)	3.0 mg/kg/day (n = 10)	15.0 mg/kg/day (n = 10)
Maternal weight (g)	334.30 ± 10.22	332.90 ± 5.88	320.60 ± 11.30	336.10 ± 4.89
Uterus weight at term (g)	59.22 ± 3.31	58.13 ± 4.29	57.72 ± 8.05	62.14 ± 3.99
Maternal weight a term minus uterus weight(g)	275.07 ± 8.98	274.76 ± 4.05	262.88 ± 5.36	273.95 ± 3.81
Fetal weight (g)	3.31 ± 0.04	3.30 ± 0.02	3.43 ± 0.03	3.30 ± 0.02
Placental weight (g)	0.50 ± 0.007	0.48 ± 0.005	0.52 ± 0.005	0.49 ± 0.006
Litter weight (g)	3.38 ± 0.07	3.34 ± 0.05	3.45 ± 0.07	3.29 ± 0.05
Number of fetuses	10.60 ± 0.68	10.30 ± 0.77	9.90 ± 1.47	10.70 ± 0.71
Number of implantations	11.40 ± 0.79	12.20 ± 0.35	11.10 ± 1.12	11.90 ± 0.60
Number of corpora lutea	12.80 ± 0.62	12.70 ± 0.36	12.00 ± 0.88	13.00 ± 0.53
% preimplantation loss	11.03	3.87	8.95	8.38
% postimplantation loss	6.54	15.84	16.07	11.36

There was no significant differences (One-way ANOVA).

placental weight among control and experimental groups. No external abnormalities were observed.

Anatomopathological analyses of maternal liver, kidneys, thyroid and adrenal glands showed a dose-dependent presence of vacuolation (Figures 2, A,B,C and D) in all tissues.

No skeletal malformations were detected on any group treated with the AQE (Table 2). Litters prenatally exposed to 3,0 and 15.0 mg/kg/day of *I.carnea* AQE presented a significant increase in skeletal abnormalities when compared to the control

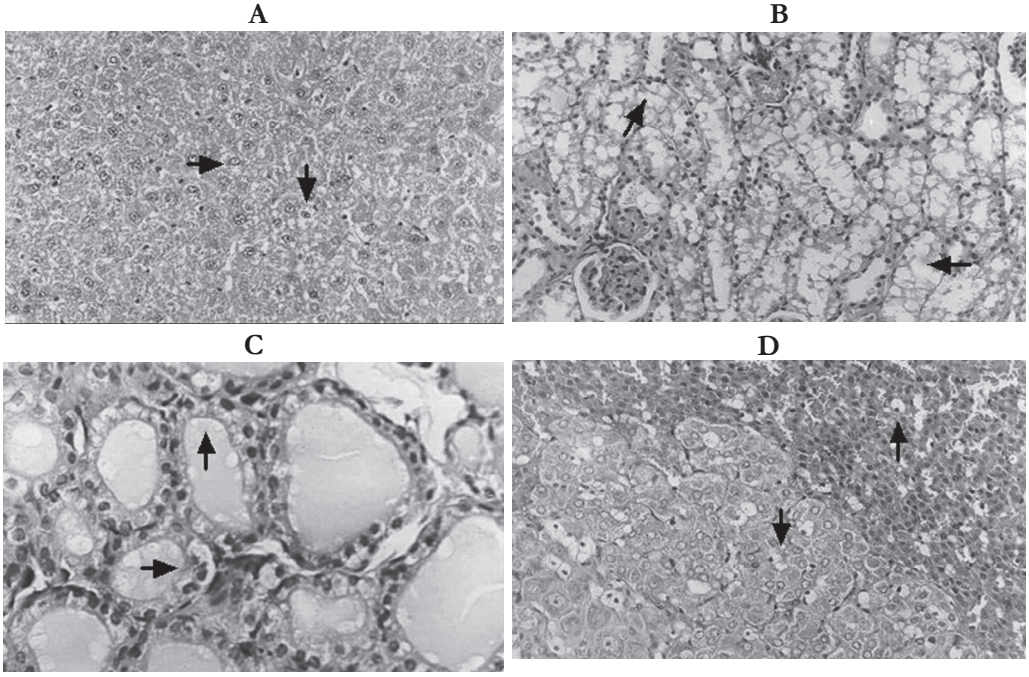

Figure 2 - (A) Photomicrograph of the liver (A), kidney (B), thyroid (C), and adrenal gland (D) of rats treated with 15.0 mg/kg of *I. carnea* AQE. Note the vacuolation caused by the treatment (arrows); H and E staining, 200X magnification

Table 2 - Skeletal malformations and abnormalities in fetuses exposed to 0.7, 3.0 or 15.0 mg/kg of *I. carnea* AQE

	Control group	0.7 mg/kg	3.0 mg/kg	15,0 mg/kg
Litter size	10	10	10	10
Number of fetuses	53	52	52	56
Skeletal malformations				
Fetuses affected	0	0	0	0
Litters affected	0	0	0	0
Skeletal abnormalities				
Fetuses affected	38	43	47	49*
Litters affected	9	10	10	10
Craniofenestriae/fetuses	0	2	4	15*
Enlarged Fontanel/fetuses	5	13	15*	23*
Sternebrae abnormalities/fetuses	38	39	42	45
Vertebral abnormalities/fetuses	0	3	10	11*
Ribs abnormalities/fetuses	0	2	1	1

*p< 0.05, compared to the control group, χ^2 Square test

group. Craniofenestriae (p< 0.0001) and vertebra abnormalities (p = 0.015) were more frequently observed after 15 mg/day AQE exposure than in control animals. An enlargement of fontanel was detected in fetuses treated with 3.0 (p=0.0135) and 15.0mg/kg/day (p=0.002) of AQE. No differences were observed among groups in regard to sternebrae or ribs.

Examination of live fetuses for ossification centers yielded significant results (Figure 3). A significant decrease in metacarpal (F(3/209) = 3.124, p=0.027), caudal vertebra (F (3/209) = 11.448, p< 0.0001), and total ossification (F (3/209) = 5.067, p = 0.002) were observed, indicating differences in metacarpal ossification on 0.7 and 15 mg/kg of *I. carnea* extract-treated animals. A reduced degree of caudal vertebra, as well as in total ossification were detected in 15 mg/kg *I. carnea* extract-treated animals, compared to control, 0.7 or 3.0 mg/kg-treated groups. No differences were observed in the remaining parameters.

Several visceral abnormalities, such as kidney symmetry, dilated renal pelvis, hemorrhagic kidney, dilated cerebral ventricle, hemorrhagic cerebrum, hemorrhagic thyroid gland (petechiae), and spongy lung were observed in fetuses prenatally treated with *I. carnea* AQE (Table 3). Thus, comparing to the control group, a significantly increased number of visceral abnormalities (p = 0.014), dilated cerebral ventricle (p = 0.016), and kidney symmetry (0.014) were detected after 15 mg/kg/day of *I. carnea* AQE. Moreover, 0.7 mg/kg/day *I. carnea* AQE-treated fetuses presented increased frequency of dilated cerebral ventricles in relation to the control group. No differences were observed among groups in the remaining parameters of visceral examination.

Discussion

We show here that exposure to *I. carnea* AQE during gestation induced maternal toxicity, represented by cytoplasmic vacuolation in several organs of the dams, suggesting that exposure of dams to *I. carnea* AQE, during 15 days of pregnancy, induce several signs of toxicity in the liver, kidneys, thyroid and adrenal glands. No visceral or skeletal malformations were detected; nonetheless, several visceral and skeletal abnormalities were observed, particularly after exposure to the higher dose of AQE. Our results show that administration of different doses of *I. carnea* AQE did not alter food and water ingestion.

However, little effects were observed in maternal performance at the end of pregnancy. In fact, there was no significant difference in number of implantation sites, number of resorptions, number of live

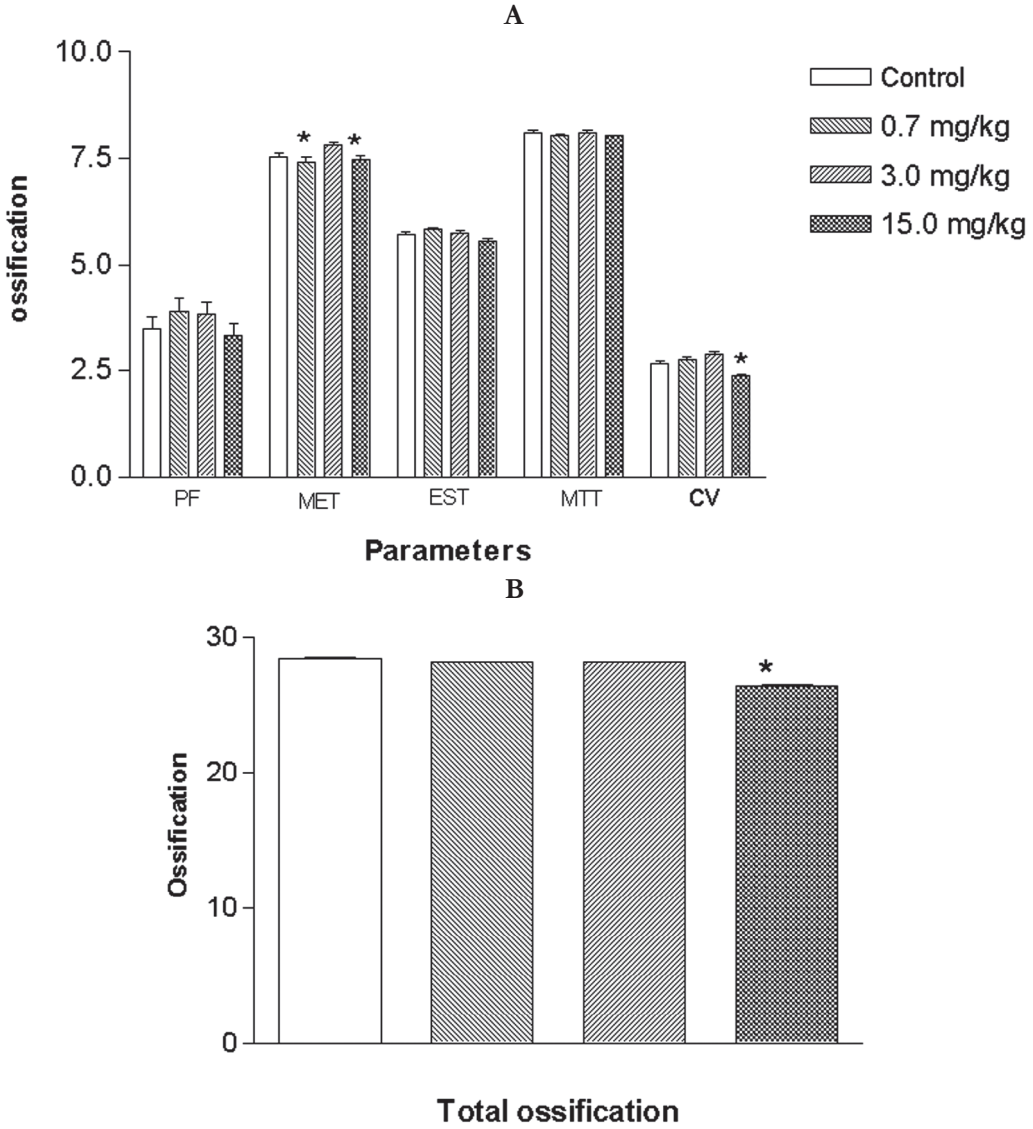

Figure 3 - Number of ossification centers (A), and total ossification centers (B) in rats exposed during pregnancy to 0.7, 3.0 or 15.0 mg/kg of *I. carnea* AQE. APF = previous phalange, MET = metacarpal ossification, EST = esternebrious, MTT = metatarsal ossification, CV = caudal vertebra. Data are presented as means ± S.E.M.

fetuses per litter, number of corpora lutea, and fetal weight or placental weight among groups. Additionally, no intrauterine effects were detected in fetal growth. Thus, this damage is also determined by the duration of exposure.[16] In our experiments *I. carnea* AQE was administered for a short period of time, i.e., 15 days. Thus, the lack of toxic effects on the placenta and fetuses may be due to both the low ability of *I.carnea* AQE active principle in crossing the placental barrier, a consequence of its hydrophilic properties, and to the short period of exposure.

Protein, carbohydrate, or lipid metabolism dysfunction, as well as changes in kidney clearance, or in hepatic and endocrine function in dams can induce embryotoxicity *per se*, leading to developmental abnormalities[17], and disrupting maternal homeostasis. Present data showed a dose-dependent vacuolation in

Table 3 - Visceral malformations and abnormalities in fetuses exposed to 0.7, 3.0 or 15.0 mg/kg of *I. carnea* AQE

	Control group	0.7 mg/kg	3.0 mg/kg	15.0 mg/kg
Litter size	10	10	10	10
Number of fetuses	51	50	46	48
Visceral malformations				
Fetuses affected	0	0	0	0
Litters affected	0	0	0	0
Visceral abnormalities				
Fetuses affected	14	18	14	25*
Litters affected	5	8	7	9
Kidney symmetry/fetuses	14	17	13	25*
Kidney pelvis enlarged/fetuses	13	16	14	18
Kidney hemorrhage/fetuses	2	0	0	0

*p< 0.05, compared to the control group, χ^2 Square test

liver, kidneys, thyroid and adrenal glands, suggesting an interference with nutrient absorption in dams exposed to *I. carnea* AQE, resulting in cellular damage in these organs.

Hueza et al. [18] showed that the body weight of pups from dams treated with the *I. carnea* AQE was diminished immediately after birth, when compared with those from young rats of untreated mothers. One hypothesis for reduced body weight of the experimental pups is due to the direct toxic effect of swainsonine in the fetuses. However, considering that it is well known that the nutritional status of mothers affects embryonic and fetal development in rats. [19,20] In the latter third period of gestation, Swainsonine has free circulation into fetal digestive and respiratory tracts.[21] Taken as a whole, the data obtained in this study revealed that the toxic principle of *I. carnea* passes the placental barrier affecting fetus development and even when *I. carnea* administration is withdrawn, swainsonine is still excreted into milk.[18]

In our study, examination of live fetuses prenatally exposed to *I. carnea* AQE aiming at the evaluation of ossification centers indicates decreased metacarpal, caudal vertebra and total ossification, mainly after the higher dose of *I .carnea*. This parameter has been used to determine the degree of fetal development.[13] Furthermore, increased skeletal and visceral abnormalities, as well as in visceral malformations, have been found in *I. carnea* AQE-exposed animals, particularly after the higher AQE dose. These data suggest that *I.carnea* AQE crosses the placental barrier, inducing direct embryotoxic effects that could be attributed to the presence of swainsonine in the plant. However, caligestines - another active principle of *I .carnea* AQE - could be partially responsible by these effects. In fact, calistegines, by an inhibitory effect on glicosydases, prevent oligosacharide and glycoprotein synthesis; therefore, both calistegines interfere with proper and adequate cell growth.[22]

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Efeitos embriotóxicos do tratamento pré-natal com extrato aquoso de *Ipomoea carnea* em ratos

Palavras-chave:
Ipomea.
Teratogênicos.
Cuidado pré-natal.

Resumo

Os efeitos embriotóxicos da exposição diária pré-natal a 0,0, 0,7, 3,0 ou 15,0mg/kg do extrato aquoso da *I. carnea* nos dias 5 a 21 de gestação foram estudados. Foram avaliados a performance reprodutiva materna, anormalidades esqueléticas e viscerais e malformações. Além disso, após o tratamento foram encontrados achados anatomopatológicos. Em relação às ratas mães, nossos resultados mostraram que a exposição às diferentes doses não afetou o peso corporal, ganho de peso, consumos de água e ração e performance reprodutiva. Apesar disso, apresentaram vacuolização citoplasmática de forma dose-dependente em fígado, rins, tireóide e glândula adrenal. Exames fetais não mostraram anormalidades externas ou malformações, sendo somente encontradas evidências de anormalidades esqueléticas e viscerais após altas doses do extrato. Foi observada redução dos centros de ossificação. Os presentes dados mostram que a ingestão prenatal do extrato de *I. carnea* induz embriotoxicidade. Estes efeitos são atribuídos à ação na homeostase maternal ou diretamente na concepção.

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Exemplo de periódico
2 KOTZEKIDOV, P.; BLOUKAS, J. G. Effect of protective cultures and packaging film permeatibility on shelflife of sliced vacuum-pocked cooked ham. **Meat Science**, v. 42, n. 3, p. 333-345, 1996.

Exemplo de livro
2 HALLIWELL, R. E. W.; GORMAN, N. T. **Veterinary clinical immunology**. London: W. B. Saunders, 1989. 548 p.

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3 FENNER, W. R. Avaliação neurológica dos pacientes. In: ETTINGER, S. J. **Tratado de medicina interna veterinária**. 3. ed. São Paulo: Manole, 1992. p. 577-606.

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4 THORTON, H. Deleterius changes in meat. In: THORTON, H. **Aspects of meat inspection**. London: Thindall & Cassel, 1973. p. 63-72.

Exemplo de tese
5 BIRGEL, E. H. **Estudo do quadro eritrocitário de caprinos (*Capra hircus*, L.) normais criados no Estado de São Paulo**: influências de fatores raciais, sexuais, etários e alimentares. 1973. 92 f. Tese (Livre Docência) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 1973.

Exemplo de evento
6 OLIVEIRA, C. A. Hormonoterapia em cadelas e gatas. In: CONGRESSO BRASILEIRO DE REPRODUÇÃO ANIMAL, 9., 1991, Belo Horizonte. **Anais...** Belo Horizonte: Colégio Brasileiro de Reprodução Animal, 1991. p. 100-111.

Exemplo de livro eletrônico
7 POORE, M. H. **Alternative feeds for beef cattle**. North Carolina: North Carolina Corporative Extension Service, 1994. Disponível em: <http:// www.ces.ncsu.edu/drought/dro-28.html>. Acesso em: 23 abr. 2007.

Exemplos de artigos de periódicos eletrônicos
8 MENDONÇA JR., C. X.; MARTINS, A. P.; MORI, A. V.; SILVA, A. B.; MORI, C. S. Efeito da adição de óleo de peixe à dieta sobre o desempenho e níveis de lípides plasmáticos e de colesterol no ovo de galinhas poedeiras. **Brazilian Journal of Veterinary Research and Animal Science**, v. 37, n. 1, 2000. Disponível em: <http://www.scielo.br/ cgi_bin/wxis.exe/iach/scielo>. Acesso em: 31 jan. 2001

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