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Impaired female sexual behavior of rat offspring exposed to *Solanum lycocarpum* unripe fruits during gestation and lactation: Lack of hormonal and fertility alterations

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Abstract

Solanum lycocarpum St. Hil (Solanaceae) is an invasive and native shrub very common in the Brazilian savanna. It is well known that this plant contain steroidal glycoalkaloids that can be transformed into an intermediate for steroidal drugs production, like oral contraceptives. In this way, it is very possible that these glycoalkaloids and its aglycone, once in the body by ingestion of *S. lycocarpum* fruits, may act disrupting to the endocrine system. Rat offspring were exposed to *S. lycocarpum* unripe fruits (10% in the diet) from gestational day (GD) 06 to post-natal day (PND) 07. The female exposed offspring showed, at adult age (PND 100), impaired sexual behavior. However, the fecal hormonal metabolite levels, measured at PND 30, PND 60 and PND 90, and the fertility (PND 120) of male and female experimental offspring were normal. We can assume that the steroidal glycoalkaloids, solamargine and solasodine, present in the fruit, are degradated, once inside the organism, to the steroidal alkaloid solasodine, which may penetrate, by simple diffusion, the placental and/or the hematoencephalical barriers and impact the fetuses. Finally, *S. lycocarpum* fruit may act as phytohormones, promoting perhaps some neural alterations that at adult age may impair the sexual behavior of the experimental female without impairing the fertility and sexual hormone synthesis. These changes observed can be the direct consequence of the toxic actions of the steroidal alkaloid on the female offspring during fetal development.

Keywords: *Solanum lycocarpum* fruit; Solasodine; Solasonine; Solamargine; Toxicology; Perinate; Phytohormones

1. Introduction

Solanum lycocarpum, St. Hil (Solanaceae), is a native shrub from the Brazilian savanna (Lorenzi, 1991). First used by native Brazilian Xavantes as an hypoglycemic agent, its fruits have been employed in folk medicine for the control of diabetes and obesity as well as for reducing cholesterol levels (Dall'Agnol and von Poser, 2003; Oliveira et al., 2003).

Solamargine and solasonine (Fig. 1) are two major glycoalkaloids found in the *S. lycocarpum* fruit and their steroidal aglycone—solasodine—is a key starting substance for the manufacture of steroidal drugs, like contraceptives, and also anti-inflammatory and diuretic agents. Solasodine can, for example, provide 16-dehydropregnenolone acetate from which dehydroepiandrosterone acetate, a key starting source for the androgen production, is obtained (Goswani et al., 2003).

Solasodine, the aglycone portion of the two glycoalkaloids, penetrates the cell membrane and reversibly binds to sterol. When the number of these alkaloid/sterol complexes rises, the extent of the impairment directly depends on the

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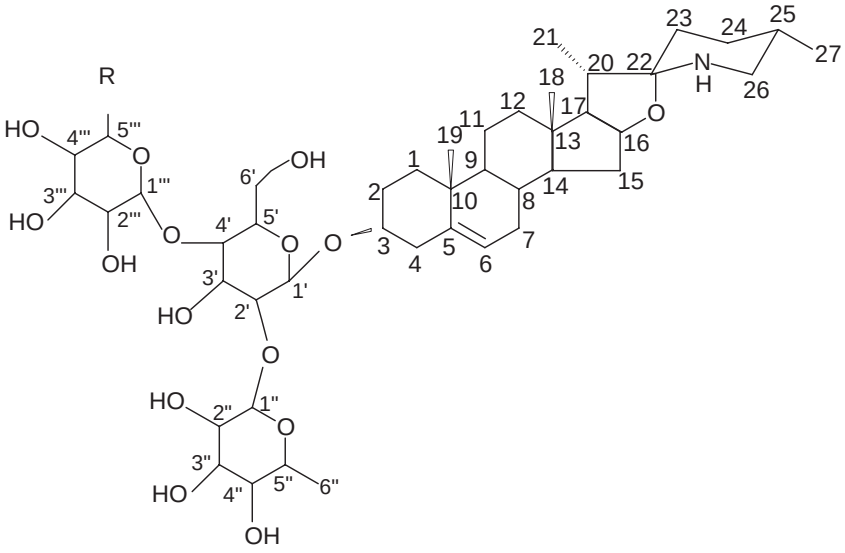

Fig. 1. Solamargine (R═CH_3) and solasonine (R═CH_2OH) chemical structure.

glycoalkaloids concentration ingested and the cholesterol type and concentration available inside the cell (Keukens et al., 1996). Solasodine, as shown above, can penetrate the cell membrane by simple diffusion and, once inside, it can exert its inhibitory action on the enzymatic conversion of dihydrolanosterol into cholesterol (Kusano et al., 1987). It is well known that cholesterol is the basic and major component for the endogenous synthesis of steroidal hormones like testosterone, estradiol and progesterone. If solasodine—and consequently the glycoalkaloids solamargine and solasonine—inhibits the cholesterol production in the organism, these components can be considered endocrine disruptors since they cause homeostasis deregulation (Kavlock, 1996).

During gestation, the plant caused slight toxicological effects in the fetuses, when pregnant rats ingested its fruits at 3% in their diet during organogenesis or preimplantation period (Maruo et al., 2003). The placental weight of the group treated during organogenesis was decreased and its fetuses showed increased sternebra anormalities; the preimplantation offspring treated group showed increased olfactory bulb hemorrhage. The actual study was undertaken to extend these previous findings, evaluating the possible effects of *S. lycocarpum* unripe fruit ingestion at 10% in the diet of pregnant dams on reproductive aspects, like sexual hormone levels, fertility capability and sexual behavior of their offspring at adult age.

2. Materials and methods

2.1. Plant

The lobeira fruit used in this study was collected in Itajubá City (Minas Gerais-Brazil) and authenticated by Prof. Dr. João Rubens Pirani from Instituto de Botânica da Universidade de São Paulo, where voucher specimens are kept in the HSF herbarium (registration Aline Schwarz, #01).

2.2. Animals

Pregnant Wistar rats from our colony weighing approximately 190–240 g each were used (GD 01=spermatozoa in the vaginal smear). The dams were individually housed in plastic cages (40 × 50 × 20 cm) at controlled room temperature (20 ± 3 °C) on a 12/12h light–dark cycle (lights on at 6:00 a.m.). The dams were randomly distributed into control ($n = 15$) and experimental ($n = 15$) groups. The treated group received chow mixed with 10% of dry milled unripe fruits of *S. lycocarpum* from GD 06 to PND 07. On PND 01, all the litters were examined externally and sexed. Litters were organized in groups of eight pups each, four males and four females, and the remaining pups were discarded. On PND 21 the offspring were weaned and the littermates separated and housed together by sex. Water and food were freely available. 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).

2.3. Preparing *S. lycocarpum* fruits to feed pregnant rats

S. lycocarpum unripe fruits were sliced and oven-dried at 50 °C. They were then milled and stored at −20 °C in dark plastic bags, protected against humidity and light.

Every week regular chow was mixed with 10% of dried and milled *S. lycocarpum* fruits, pelletized and stored in paperboard bags in a temperature-controlled room (22–25 °C). The experimental female rats were fed this pelleted diet, containing 10% of dried *S. lycocarpum* fruits, from GD 06 to PND 07.

In this study it was observed that a pregnant rat ingest by day approximately 20–25 g of chow. So, chow with 10% of *S. lycocarpum* fruit will permit the intake of approximately 2–2.5 g fruit per day, the same as 2 g fruit/animal/day or 8 g fruit/kg/day, if one pregnant rat weight approximately 250 g.

2.4. Fertility tests

The purpose of these tests is to evaluate possible alterations in the offspring fertility by the observation of egg production and ovulation (by females), egg fecundation capability (by males sperm), embryonic fixation to endometrium, fetuses development and losses during pregnancy, and size and weight of the litters as examples of the range of parameters evaluated.

2.4.1. In female

At PND 100, after body weight recording, 8 females in each group, from distinct litters and housed individually, were allowed to mate with males of our stock, previously tested as fertile, for a maximum period of 15 days. Only one male was allowed per cage. The pregnancy GD 01 was determined the day spermatozoa was found in the vaginal smears. The males were removed from the cages and the females received food and water freely during pregnancy. On GD 21, after body weight recording, the females were euthanized by inhalation of carbon monoxide. Laparotomy in each female, by longitudinal incision at the abdomen wall, showed the uterine horns and fetuses. By counting the number of implantations and reabsorptions in the uterine horns and the number of corpus luteus in the ovaries, preimplantation losses = [(number of corpus luteus − number of implantations)/number of corpus luteus] × 100, post-implantation losses = [(number of implantations − number alive fetuses)/number of implantations] × 100, implantation percentage = (number of implantations/number of corpus luteus) × 100 and percentage of living fetuses = (number of alive fetuses/number of implantations) × 100, were calculated. The litter size and weight, number and weight of male and female pups, and uterus and ovary weight from each female were also evaluated.

2.4.2. In male

At PND 100, 8 males in each group, from distinct litters, were housed individually. Four naive female adult rats from our stock were placed in each cage, for a period no longer than 15 days. Pregnancy was determined by the presence of spermatozoa in the vaginal smears, when GD 01 was established. Females without spermatozoa in vaginal smears during the 15-day period were discarded. The pregnant ones received food and water freely. On GD 21 the females were euthanized by carbon monoxide inhalation. Laparotomy was carried out and the same parameters for the female reproductive performance study were applied.

2.5. Measurement of sexual hormones in feces

On PND 30, PND 60 and PND 90, feces of male and female control and exposed offspring (n = 7/group) were collected. Portions of 0.25 to 0.30 g were extracted, applying an existing technique (Brown et al., 1994). In glass assay tubes the portions were mixed and heated for 25 min with 5 mL 90% ethanol. After homogenization in vortex and centrifugation at 500 $\times g$ for 15 min the supernatants were transferred to another glass assay tube and reserved. The pellets were again homogenized with 5 mL of 90% ethanol and the same procedures described above were repeated. The two supernatants were joined, dried completely and then redissolved in 1 mL of methanol. The solution was maintained in a freezer at −20 °C prior to analysis.

The hormone fecal metabolite levels were quantified by radioimuno assay (RIA), validated for male and female rat feces, by employing the depleted matrix parallel method that shows if the hormones in the feces are interacting with the antibody of the hormone kits, similar to the standard hormone.

The RIA primary concentrations were expressed as nanogram/milliliter for progesterone metabolites, picogram/milliliter for estradiol metabolites and nanogram/deciliter for testosterone metabolites. The results were all converted by the expression: $FC = [(C \times V_f \times D \times 1)/Wi]/1000$, where: FC = final concentration; C = RIA concentration; V_f = feces final volume at the end of the extraction phase; D = dilution employed; and W_i = initial weight of feces. Also, to improve and facilitate the data observation, the progesterone and estradiol values obtained were converted to nanogram/gram and the testosterone values obtained were converted to microgram/gram.

2.6. Sexual behavior studies

For male and female sexual behavior evaluation the animals were housed in a room with reversed light/dark cycle for at least 21 days (time required for organism adaptation). During observation, a lamp of 40 W provided room illumination with a red filter. A rectangular wood-made gray painted box (56 cm long × 35 cm wide × 31 cm high) with a moveable cover and frontal glass wall was employed. The inner side of the box was covered with a 3.0 cm sawdust layer.

2.6.1. Female sexual behavior

At PND 75, others 15 females of each group, obtained from distinct litters, were ovariectomized. From the total, 10 control and 11 experimental females survived the surgical procedure and were left untouched in their cages (3 to 4 females per cage) in a room with reversed light/dark cycle for 21 days, a period sufficient for metabolization and excretion of all steroidal sexual hormones and its metabolites. After the habituation period the

Table 1
Fertility evaluation of male offspring at PND 120

Parameters evaluated	Control	Experimental
Litter weight (g)	31.9±1.9	33.6±2.6
Total number of fetuses per litter	9.4±0.6	9.3±0.6
Number of male fetuses per litter	5.1±1.5	4.8±0.5
Number of female fetuses per litter	4.3±0.4	4.4±0.4
Male fetuses body weight (g)	3.5±0.1	3.8±0.1*
Female fetuses body weight (g)	3.4±0.1	3.5±0.1
Uterus weight (g)	4.0±0.1	3.9± 0.1
Right ovary weight (g)	0.07±0.01	0.07±0.01
Left ovary weight (g)	0.07± 0.01	0.07±0.01
Number of corpus luteus in right ovary	6.8±0.4	6.4±0.4
Number of corpus luteus in left ovary	5.5±0.4	6.5±0.4
Number of implantations	10.7±0.6	10.5±0.7
Number of reabsorptions	2.4±1.1	3.5±1.5
Pre implantation lost (%)	12.9±3.2	17.7±4.7
Post implantation lost (%)	12.7±4.0	11.9±2.9
Implantation (%)	87.1±3.2	82.4±4.7
Alive fetuses (%)	83.4±5.2	88.1±2.9

Data are expressed as mean±SEM. ($n=8$/group).
* $p<0.05$—two-way ANOVA followed by Tukey–Kramer test.

females, now with PND100, received 50 μg/kg 17-β-estradiol (Sigma) and 2 mg/kg progesterone (Sigma), 54 and 6 h before the test, respectively. At the moment of the test, realized during the dark cycle, the females were in an induced pseudo-estrous phase and, therefore, sexually receptive for adult experience males of our stock. In the mating box the male was allowed to adapt for 5 min before one rendered sexually receptive female was introduced. The quotient of lordosis (QL=number of lordosis/number of mounts × 100) was calculated by the presence or absence of lordosis, position that allows penial intromission, in 10 mounts realized by the male. This technique was based on the description by Beach (1967).

2.6.2. Male sexual behavior

On PND 100, another 12 males of each group, obtained from different litters, were employed. The male was placed in the mating box 5 min before introducing an ovariectomized female, which was rendered sexually receptive like described above. If the male did not mount within the next 10 min, the test was considered negative and the animal was discarded. The following measures were recorded at a 40 min session: mount and intromission latencies, defined as the times from introduction of the female in the cage to the first mount and intromission, respectively; mount and intromission frequencies, the number of mounts and intromissions preceding the first ejaculation; ejaculation latency, the time from introduction of the female in the cage to first ejaculation; both mount and intromission latencies post-ejaculation, the time for the first mount and intromission after the first ejaculation; intromission frequencies post-ejaculation, the number of intromissions after the first ejaculation; and total number of ejaculations. This technique was based on the description by Bitran and Hull (1987) for evaluation of sexual motivation. Hand-operated counters and stopwatches were employed to score these parameters.

The parameters copulatory efficiency (Malmnas, 1973), frequency of mounts and of intromissions per minute (Bitran and Hull, 1987) and sexual activity score (Agmo, 1997) were also evaluated for male rats sexual potency study.

2.7. Statistical analyses

The fertility capability and the hormonal data were analyzed by the two-way analysis of variance (ANOVA). Sexual behavior parameters were analysed by the t test. When significant values were detected in the parameters evaluated by the ANOVA, the Tukey–Kramer test was employed as a post-hoc test. In all cases, the level of significance was set at $p<.05$.

3. Results

3.1. Fertility tests

The two-way ANOVA employed to evaluate the fertility of males (Table 1) and females (Table 2) showed few alterations provenient from factor treatment, with no interaction between factors treatment and sex, on the following parameters: reduced number of corpus luteus in the right ovary [$F(1/1/28)=1.117$, $p=0.0286$], reduced preimplantation losses [$F(1/1/28)=3.384$, $p=0.0308$] and higher percentage of living fetuses [$F(1/1/28)=3.212$, $p=0.0332$] in the treated females; also, higher experimental male fetal body weight at PND 01 [$F(1/1/28)=5.465$,

Table 2
Fertility evaluation of female offspring at PND120

Parameters evaluated	Control	Experimental
Litter weight (g)	32.4±3.1	35.5±3.0
Total number of fetuses per litter	9.6±1.0	10.1±0.6
Number of male fetuses per litter	4.9±0.6	4.0±0.7
Number of female fetuses per litter	4.8±0.8	6.1±0.9
Male fetuses body weight (g)	3.5±0.1	3.8±0.5
Female fetuses body weight (g)	3.4±0.14	3.6±0.4
Uterus weight (g)	3.9±0.1	3.9±0.1
Right ovary weight (g)	0.07±0.01	0.07±0.01
Left ovary weight (g)	0.07±0.01	0.08±0.01
Number of corpus luteus in right ovary	7.3±0.6	5.3±0.6*
Number of corpus luteus in left ovary	7.6±1.6	6.1±0.5
Number of implantations	11.2±0.6	10.6±0.5
Number of reabsorptions	0.4±0.2	0.3±0.2
Preimplantation lost (%)	19.9±9.4	7.9±3.3*
Post-implantation lost (%)	12.5±3.1	5.0±1.8
Implantation percent	80.±9.4	92.1±3.3
Percentage of living fetuses	84.5±3.1	94.9±1.8*

Data are expressed as mean±SEM. ($n=8$/group).
* $p<0.05$—two-way ANOVA followed by Tukey–Kramer test.

$p=0.0316$]. The Tukey–Kramer test employed as a post-hoc test confirmed the reduced number of corpus luteus in the right ovary ($p<0.05$), the reduced preimplantation losses ($p<0.05$) and the higher percentage of living fetuses ($p<0.05$) in the treated females when compared to the control ones; also, increased experimental male fetal body weight at PND 01 ($p<0.05$) when compared to control male fetuses was observed.

3.2. Fecal metabolite hormone measurement

The concentration levels of metabolites of the sexual hormones estradiol, progesterone and testosterone, in feces, of male and female offspring at PND 30, PND 60 and PND 90 are presented in Fig. 2A, B and C, respectively. The two-way ANOVA revealed no alterations, due from factor treatment, in the concentration levels of fecal estradiol metabolites at the analyzed periods of the exposed male and female offspring but showed alterations caused by factor sex (sexual dimorphism), without interaction with factor treatment, on PND 60 [$F(1/1/24)=23.029$, $p<0.001$] and PND 90 [$F(1/1/24)=9.861$, $p=0.005$], where female offspring showed increased concentrations of fecal progesterone metabolites when compared to male offspring. Likewise, the two-way ANOVA showed alterations caused by sexual factors (sexual dimorphism), with no interaction between both factors treatment and sex, on PND 60 [$F(1/1/24)=15.847$ $p<0.001$] and PND 90 [$F(1/1/24)=19.333$, $p<0.001$], where male offspring showed increased fecal testosterone metabolite levels when compared to female offspring. The Tukey–Kramer test employed as a post-hoc test revealed in experimental females, at PND 60, increased levels of estradiol metabolites ($p<0.05$) when compared to control ones.

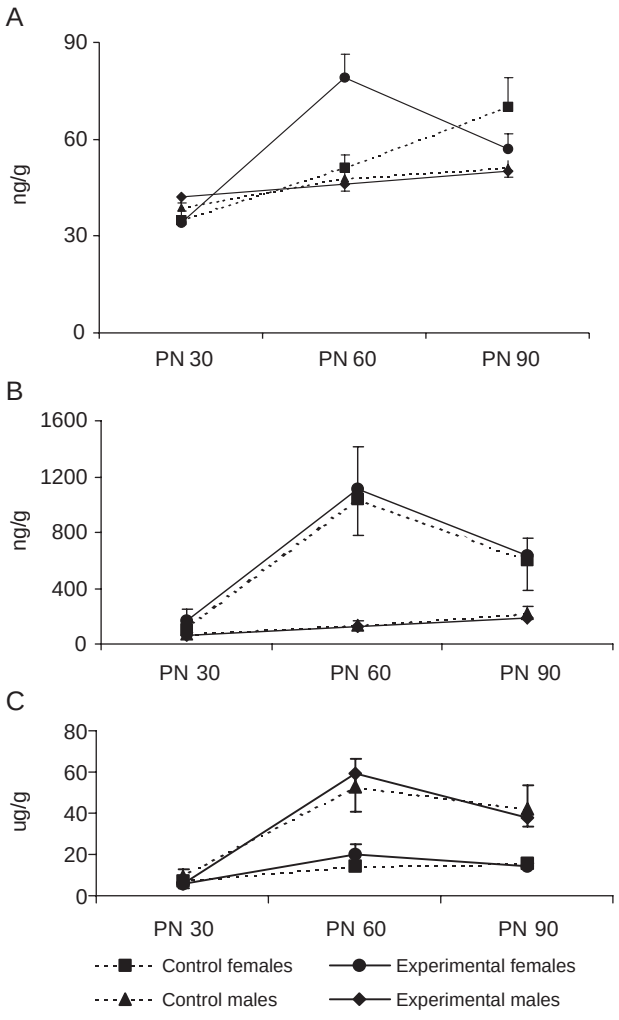

Fig. 2. Fecal metabolite levels of the sexual hormones estradiol (A), progesterone (B) and testosterone (C) of male and female offspring on PND 30, PND 60 and PND 90. Data are expressed as mean±SEM. ($n=7$/group). *$p<0.05$, ANOVA followed by Tukey–Kramer post-hoc test.

Table 3
Female offspring (PND 100) lordosis sexual behavior (lordosis number/mounts number × 100) of rats treated during gestation and lactation periods (GD 06 to PND 07) with 10% of *S. lycocarpum* fruits in the diet

Groups	Lordosis behavior (%)
Control ($n=10$)	74.0±6.2
Exposed ($n=11$)	48.2±9.2*

Data are presented as means±SEM.
* $p<.05$ (Student t test).

3.3. Sexual behavior studies

The t test revealed reduced coefficient of lordosis in experimental females [$t=2.276$, $F(1/19)=2.450$, $p<0.05$] when compared to control females. Table 3 presents the medians and respective standard errors. No alterations were observed in all the parameters evaluated (Table 4) in the male sexual behavior test.

Table 4
Male offspring sexual behavior (PND 100) of rats treated, during gestation and lactation periods (GD 06 to PND 07), with 10% of *S. lycocarpum* fruits in the diet

Parameters	Control	Exposed
Latency for 1st M (seconds—s)	31.3±7.4	56.7±14.3
Latency for 1st I (s)	42.8±8.4	68.9±57.4
Latency for 1st ejaculation (s)	1417.4±98.5	1602.9±109.8
Number of M until ejaculation	10.7±2.3	12.2±2.2
Number of I until ejaculation	51.5±6.7	47.4±5.2
Post-ejaculation M latency	311. 7±25.9	314.9±12.2
Post-ejaculation I latency	312.7±28.4	315.4±14.1
Total M in 40 min	13.2±2.2	14.5±2.6
Total I in 40 min	73.5±5.2	66.3±7.3
Total number of ejaculations	1.7±0.2	1.3±0.2
Sexual activity score	16.1±0.3	15.3±0.5
Mounts/minute frequency	59.9±8.5	58.5±10.8
Intromissions/minute frequency	324.9±23.9	282.3±50.3
Copulatory efficiency	85.4±1.9	81.7±2.6

$n=12$/group.
Data are presented as means±SEM. (Student t test).
M=mount and I=intromission.

4. Discussion

Our results indicate that the introduction of 10% of *S. lycocarpum* fruits in the diet, from GD 06 to PND 07, did not impair gestation or intrauterine fetuses growth in the exposed offspring at adult age.

Pup body size and weight at birth are important variables that may alter the physical development of the animals (Leonard, 1982). The exposed intrauterine offspring, here employed for the evaluation of fertility, fecal metabolite levels of sexual hormones and sexual behavior, showed, in previous study, reduced body length and normal body weight at birth when compared to the control offspring. These findings can be at least in part explained by the two main properties of solasodine: its ability to interact with the membrane sterols and disrupt membrane integrity and function (Roddick et al., 2001) and its inhibitory effect on the enzymatic conversion of dihydrolanosterol into cholesterol (Kusano et al., 1987). The male experimental second generation showed higher body weight of male fetuses while no alterations were observed in the female body weight second generation, showing that the glycoalkaloids promote lower body weight only when the animal is exposed to the fruit.

The steroidal alkaloid solasodine and its glycoalkaloids solamargine and solasonine, in *S. lycocarpum* fruit, are plant-derived compounds that structurally or functionally mimic estrogens like a wide variety of phytoestrogens (Ososki and Kennelly, 2003). The fruits, however, showed a weak toxic activity in the female pups when given to pregnant rats at 3% in their diet during organogenesis and no toxic effects were observed in the male pups (Maruo et al., 2003). In our study, dams were treated with a higher dose (10% in the diet) for a longer period, including lactation. Male and female pups were affected in the first but not in the second offspring generation. However, even using a higher dose, it seems that the *S. lycocarpum* toxic effects were less important.

Steroidal sexual hormones are fundamental for the embryo development and sex differentiation. It is well known that the androgens play an important role in the male embryos and fetuses from the last days in the womb to about 10 days post-partum, masculinizing and defeminizing the brain, causing structural and neurochemical changes in the developing brain, which do not occur in the females due to absence of exposition to steroidal structures (McClusky and Naftolin, 1981). It is also well known that solasodine can penetrate the cell membrane by simple diffusion. Once inside the placenta and consequently inside the fetuses, this compound possibly disrupt this important hormonal action, by acting as a hormonal agent, or, simpler than that, by inhibiting cholesterol formation, disrupting the production chain of the sexual hormones since cholesterol is the key starting component for this synthesis (Kusano et al., 1987).

For evaluation of these effects, sexual behaviors of male and female offspring at adult age were observed. Experimental males showed normal sexual behavior, motivation and copulatory efficiency, while experimental females showed impaired sexual behavior, evaluated by lower lordosis behavior response. These alterations may be explained by the possible presence of solasodine in the female brain during fetus development period and/or lactation, promoting alterations in the structure or chemical of the brain, and does not occur at normal conditions, like described above, where the female fetuses brain present no contact and interaction with steroidal hormones, permitting the female aspect of their brains (McClusky and Naftolin, 1981).

Ejaculation and emission of semen to the female genital tract require effective mounting, penile erection, intromission and female receptiveness, evaluated by lordosis. For the reproductive performance study of the male offspring, the sexual behavior and the sperm quality were indirectly evaluated by observing and examining the gestation and the respective offspring obtained by mating these males and, for the female fertility study, the gestation and respective reproductive performance after mating with males were directly evaluated through observation and examination. The intrauterine control and exposed males were indirectly evaluated by maintaining the gestation and absence of preimplantation and post-implantation losses of their females. The former showed normal fertility capability when compared to the fertility of the latter. These females maintained the fecundated oocytes since the pre- and post-implantation losses were reduced.

The androgens were first considered agents that promote male sexual behavior. Now it is well known that progesterone, in some circumstances, counteracts androgens and protects female fetuses brain from the effects of androgens (Renner et al., 1987). Also, it is only after their conversion to estrogens that the androgens exert their decisive effects on the target cell nuclei, explaining why pharmacologic doses of exogenous estrogens may masculinize females of various species (Ehrhardt and Meyer-Bahlburg, 1981). In fact no alterations in the synthesis of the sexual hormones were detected since no fecal metabolite levels of sexual hormones were observed.

Having in mind the absence of effects on fertility as well as the normal sexual hormone levels of the exposed rat offspring at adult age, in a sexually active period, and also the lower lordosis behavior observed in experimental females, it seems that the *S. lycocarpum* fruits, 10% in the dam's diet during gestation and beginning of lactation, acts as phytohormones, promoting perhaps some neural alterations that at adult age may impair the sexual behavior of the experimental females without impairing the fertility and sexual hormone synthesis of the offspring. However, the changes observed may be the direct consequence of the toxic actions of the alkaloids on the female offspring reproductive system.

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