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Research Article

Evaluation of the Use of Goats as an Animal Model in Solanum malacoxylon Toxicity: Growing Goats and Prenatal Study

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Abstract

Solanum malacoxylon a toxic plant contains a glycoside conjugated to Vitamin D3 that promotes in cattle a calcinogenic disease characterized by weight loss, stiffened, painful gait, hypercalcemia, hyperphosphatemia, and mineralization of soft tissues. The aim of this study was to evaluate the toxic effects of *S.malacoxylon* on ruminant species, the goat, with similar digestive physiology and placentation to bovines, however this animal species is easier and cheaper to maintain and presenting gestation period shorter than bovines. Two trials were performed, in the first one, male growing goats were placed in two groups, experimental animals received *S.malacoxylon* (200mg/kg/BW/day). In the second trial, pregnant goats were allocated in four groups: control and three experimental that received 12.5, 25.0 and 50.0 mg/kg/BW of *S.malacoxylon*. Animals treated with the plant, from both trials, showed rigid walking, lower body weight, increase in serum calcium, phosphorus and effects on alkaline phosphatase. Kids from pregnant treated with *S malacoxylon* had lower birth weight. At necropsy was observed calcification of cardiac valves, cardiac muscle and arteries in growing goats, mothers and their kids. Histopathological study showed alteration on extracellular matrix and calcium deposition on soft tissues. These results permit to verify that goats are a good animal model for studying the plant intoxication, once it was possible to reproduce all effects described in natural condition in cattle. The toxic components of *S.malacoxylon* can affect both, dams and fetuses, from ruminants and this form of intoxication may account for the heavy losses in the cattle industry.

INTRODUCTION

Enzootic calcinosis of domestic animals has been identified by many researchers as a disease that causes heavy economic losses to the cattle industry [1,2]. This endemic disorder observed in livestock in Latin America, called “espichamento” in Brazil [3] and “enteque seco” in Argentina [4], occurs when animals graze for several weeks on leaves of the shrub *Solanum malacoxylon.* The toxicosis that develops in these animals is characterized by rapid weight loss, as well as stiffened and painful gait [4,5]. Sera analysis of intoxicated animals showed hypercalcemia, hyperphosphatemia, and extensive mineralization of soft tissue [3,4,6,7,8]. These symptoms and signals resulted from a glycoside conjugated to Vitamin D_3 (1.25 $[OH]_2D_3$) on *S. malacoxylon* leaves [9,10,11].

Animal death is due to calcinosis, i.e., pathologic deposition of calcium phosphate in several organs, resulting in weakness, malnutrition caused by the inability to move, and cardiac and pulmonary insufficiency leading to losses in the value of beef carcass. Decreases in fertility and milk production have been related to *S. malacoxylon* and may also account for the losses [12,1,13].

Previous studies performed with conventional laboratory animal models, such as rats and rabbits, showed that *S. malacoxylon* exposure causes the characteristic symptoms and signals of calcinosis [14,15]. However, a study performed with bovines, the primary affected species, is expensive due to the cost of the animals and their maintenance. These costs are much higher if the goal of the study is to perform a teratological trial, as the bovine gestation period is very long.

We studied the fetal effects of *S. malacoxylon* in rabbits [16] and rats [17], and we verified alterations in both animal species; however, we should consider that embryonic development of ruminants is distinct from that of rodents, lagomorphs and other monogastric species [18]. Thus, the purpose of the present study was to evaluate the toxic effects of *S. malacoxylon* on ruminant species, i.e., the goat, with similar digestive physiology [19] and placentation to bovines [20]. This small ruminant is easier and cheaper to maintain than bovine, given that the gestation period much shorter (150 days versus 280 days for the cow).

MATERIALS AND METHODS

This study was conducted at the University of São Paulo (USP) Experimental Station, Pirassununga, Sao Paulo state, Brazil (S21°58', W47°27'). The procedures were approved by the USP Animal Ethics Committee, and all animal care and handling was performed by experienced personnel under veterinary supervision.

Plant material

Leaves of *S. malacoxylon* collected from the Pantanal region, Mato Grosso do Sul, Brazil, were air-dried, powdered and offered to individual goats; all treated animals readily ate all offered *S. malacoxylon* during the exposure period.

Animals and experimental design

Twenty Saanen mixed-breed pregnant female goats (12 months old), ten Saanen mixed-breed castrated young male goats (60 days old) and one fertile male of the same breed (2 years old) were used in this study. Two trials were performed, and all procedures were conducted under veterinary supervision.

Trial 1

Young male goats were placed into two groups (n=5 per group): control animals received no experimental treatment, and SM-animals received 200 mg per kg of body weight (mg/kg BW) of *S. malacoxylon* dry leaves. The animals remained on this experimental regimen for 35 days. During this period, the SM-treated animals received the treatment every morning from the 1st day of experiment to the 28th. *S. malacoxylon* (as a dry powder) was added to the commercial ration. Each animal's diet was supplemented with 200 g/day of a commercial supplement: ground corn (60.6% on DM basis) and soybean meal (36%) with 3.4% mineral salt. Additionally, after the animals had consumed the commercial ration and *S. malacoxylon*, they were given free access to chopped sugar cane residue (*Saccharum officinarum* L.) sufficient for overnight feeding. The residual sugar cane was removed before feeding commenced the next day. Fresh water was available *ad libitum*. The animals were housed together in an open-air barn with a raised wooden floor, as is typical in Brazil. The males were fed individually in tie stalls when given the *S. malacoxylon* and the commercial supplement; they were group-fed the sugar cane overnight.

During the experiment, the goats were clinically evaluated. Body weight gain was measured weekly (from day 0 to day 28), and blood samples of each goat were obtained by jugular vein puncture before the plant administration and then every other week at the end of the study period. Blood serum was frozen and stored at -10°C until analysis. Commercial kits (CELM®, Brazil) were used for the determination of calcium, phosphorus and alkaline phosphatase serum levels using a CELM SBA-200 blood analyzer (CELMs®, Brazil). After the final blood sample collection, one goat from each group was euthanized and necropsied. Representative samples of lungs, heart, aorta, liver, muscles and kidneys were harvested and stored in 10% buffered formalin, and then were embedded in paraffin and sectioned at 5 µm. Sections were stained with hematoxylin and eosin stain (HE). Von Kossa stain was also used to detect calcium carbonate and phosphate deposition [21].

Trial 2

Female goats' breeding was synchronized using standard methods with vaginal pessaries, and all does were bred twice by the same fertile male of the same breed. The day of breeding was defined as day 1 of gestation, and the pregnancies were confirmed by an ultrasound (US) apparatus (Scanner 100 Vet, Pie Medical Lineal probe, 5.0/7.0 MHz) on day 27.

Pregnant goats were randomly allocated into 4 groups (n=5 per group) and received varying doses of *S. malacoxylon*, in mg/kg BW, as follows: 0 (Control group), 12.5 (SM1 group), 25.0 (SM2 group) and 50.0 (SM5 group). The treatment animals were provided with *S. malacoxylon* from the 30th day of gestation to the 90th. The method of administration of the toxic plant, animal treatment, housing, clinical evaluation and weighing were as described in trial 1.

Blood samples were collected via jugular venipuncture biweekly from the 30th day of gestation to the 135th. Blood serum was frozen and stored at -10°C until analysis. Commercial kits (CELMs®, Brazil) were used for the determination of calcium, phosphorus, magnesium, urea, creatinine and alkaline phosphatase using a CELM SBA-200 blood analyzer (CELMs®, Brazil).

Ultrasonographic evaluation was performed on each pregnant goat using established methods [22, 23]. The following fetal parameters were measured: fetal movement (FM) and heart rate (HR) on days 35, 49 and 63; crown-rump length (CRL) on days 35 and 49; and thoracic diameter (TD), abdominal diameter (AD) and biparietal diameter (BPD) on day 63. FM was evaluated for 5 minutes. The movements were counted manually, and the number of movements per minute was calculated.

At birth, each neonate's body weight was immediately recorded and the gender determined. Each kid was then examined carefully for gross abnormalities [24]. After this procedure, one mother and her kid were euthanized and necropsied to collect representative samples of tissue as in trial 1.

Statistical analysis

Serum biochemistry and ultrasound measurements were analyzed using a mixed linear model (Proc Mixed) for each treatment. The animals were nested within the treatments, and repeated measurements of the variables were taken over time. The animals were considered as a random factor in the model.

In trial 1, total weight gain was analyzed statistically by unpaired Student's *t* test. In trial 2, total weight gain and birth

weight were analyzed statistically by one-way analysis of variance (ANOVA) followed by Dunnett's test.

Data are reported as mean ± SEM and were analyzed using SAS software (Version 9.2; SAS Institute, Cary, NC). In all cases, the probability of significant differences was set at $\alpha = 0.05$.

RESULTS AND DISCUSSION

We performed trial 1 to compare the clinical picture of toxicity by *S. malacoxylon* presented in goats with that in cattle naturally poisoned with the plant. In all weeks of plant administration, goats showed a decrease in body weight gain ($p<0.05$; Table 1), which is supported by previous studies by Carrilo and Worker [4] and Döbereiner et al. [5] in bovines—the species that is naturally intoxicated by the plant. Similar to other studies showing the effect of *S. malacoxylon* in cattle [13], we observed that goats showed physical deterioration, limited mobility, rigid walk and lameness after two weeks of treatment which were maintained until the end of the experiment. Animals from both groups showed no changes in body temperature, heart and respiratory rates, ruminal movements or color of mucous membranes.

Because the main active component of *S. malacoxylon* is vitamin D, it is expected that plant intoxication may interfere with the absorption and deposition of calcium. In fact, many studies in different animal species have showed this alteration in calcium metabolism [13]. Goats from SM group also displayed hypercalcemia and hyperphosphatemia on experiment days 7, 14, 21 and 30 compared with the control group ($p<0.05$; Figure 1). Interestingly, goats presented calcium and phosphorus levels similar to those of the control animals one week after *S. malacoxylon* withdrawal.

These biochemical changes in animals treated with *S. malacoxylon* were confirmed with a histopathological study, which revealed marked mineralization of soft tissue. The heart and lungs were the most affected organs, showing intense calcification of cardiac valves and cardiac muscle coronary, aorta and pulmonary artery calcification. The histological study of the aorta collected from animals treated with the plant and stained with HE showed alterations in elastic laminae of the tunica intima. In addition, from the histological evaluation of the same stain in kidney sections from animals in the SM group, it was possible to verify matrix alteration in the basal membrane of convoluted tubules and glomerulus. When the Von Kossa technique was employed in these same histological slides, we observed phosphate and carbonate deposition in elastic laminae of the tunica intima in the aorta (Figure 2).

Table 1: Body weight gain (kg/ Mean ± S.E.M.) during plant administration of young male goats allocated in two treatment groups: control and SM (200 mg/kg body weight daily of *S. malacoxylun*, dry-air leaves from the 1st day of the experiment to the 28th). Experimental basis of 35 days and samples were collected weekly.

Groups	Week				Total
	1	2	3	4	
Control(5)[a]	1.26 ± 0.3	0.72 ± 0.2	0.45 ± 0.3	2.60 ± 0.2	5.03 ± 0.3
SM(5)	-0.25 ± 0.2*	-0.20 ± 0.2*	-0.34 ± 0.2*	-0.54 ± 0.4*	-1.33 ± 0.4*

[a]Number of goats.
*p<0.05; compared to control.

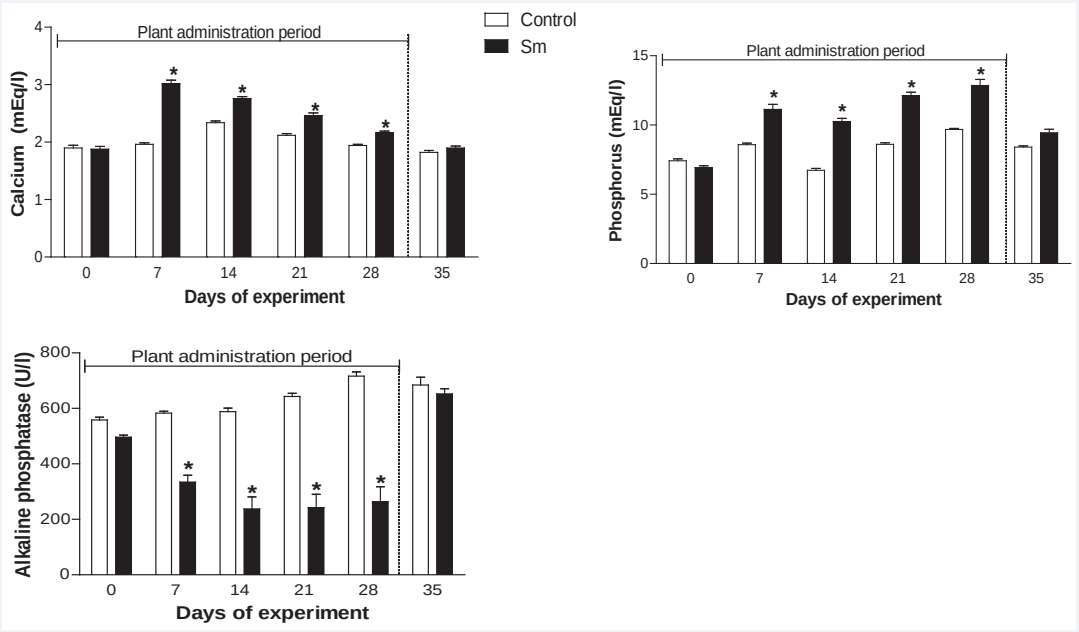

Figure 1 Serum level of calcium, phosphorus and alkaline phosphate activity of young male goats allocated in two treatment groups: control and SM (200 mg/kg body weight daily of *S. malacoxylun*, dry-air leaves from the 1st day of experiment to the 28th). Experimental basis of 35 days and samples were collected weekly (*p<0.05; compared to control).

A significant decrease in alkaline phosphatase ($p<0.05$; Figure 1) was verified in experimental goats during the entire period of plant administration (28 days), in contrast to animals that were not exposed to *S. malacoxylon*. Considering that we used growing goats in this study and that animals of this age present intense osteoclastic and osteoblastic activity [25], it is possible that the "higher levels" of this enzyme verified in the control animals were due to osseous growth and bone remodeling [25]. However, it is well known that animals exposed to *S. malacoxylon* show a significant decrease in bone reabsorption [13], and the higher concentrations of blood calcium and phosphorus in intoxicated animals originated mainly from increased intestinal absorption rather than from bone reabsorption [26]. Thus, it is possible to suggest that goats treated with *S. malacoyxlon* should present much lower activity of alkaline phosphatase than control animals. Reinforcing this assumption is the evaluation of alkaline phosphatase at the 35th day of the experiment, (i.e., one week after the last plant administration), in which no differences in this enzyme were detected between control and experimental goats.

Due to the fact that growing males poisoned at a dose of 200 mg/kg BW showed a marked reduction in weight gain, we decided to reduce the dose used in the experiment with pregnant females. This procedure was necessary because we planned to administer the plant to pregnant goats from the implantation period until delivery (i.e., approximately 120 days of *S. malacoxylon* treatment), and such a long period could cause undernutrition in the mothers and thus compromise fetal development [27]. However, even with lower doses pregnant females from the SM2 and SM5 groups had decreased body weight gain ($p<0.05$; Table 2) and dose-dependent physical deterioration, limited mobility and rigid walk after three weeks of treatment; thus, we decided to suspend the administration of the *S. malacoxylon* at the 90th day of gestation. Even after the cessation of plant administration, one female from the SM2 group in poor condition, i.e., extremely lean; difficulty standing and feeding, died in the final third of gestation. In addition, ceasing plant administration caused no improvement in body weight gain or remission of signs of intoxication in any of the pregnant goats. In contrast to pregnant rabbits [16], female goats did not show reproductive changes such as fetal death, stillbirth or abortion (Table 2). None of the females showed alterations in heart and respiratory rates, ruminal movements, body temperature or color of mucous membranes.

Pregnant females treated with *S. malacoxylon* presented hypercalcemia and hyperphosphatemia ($p<0.05$; Figure 3), which is consistent with the observations from growing goats. This result corroborates the assumption that *S. malacoxylon* interferes with bone remodeling in growing goats. Serum levels of magnesium, urea, and creatinine, as well as the activity of alkaline phosphatase showed fluctuations, but no significant differences were observed among the data from the different groups (data not shown).

Interestingly, after *S. malacoxylon* was withdrawn, there was a decrease in the serum levels of calcium in experimental mothers; however, at 140 days of gestation, the levels of this mineral was significantly lower than those found in controls ($p<0.05$; Figure 3). One possible hypothesis to explain these decreases could be that ingestion of the active form of vitamin D was ceased at 90 days of gestation, and calcium and phosphorus returned to the physiological levels of absorption. However, the correlation of limited mobility to feed with the anorexic effect produced by *S. malacoxylon* can lead to a decrease in the consumption of calcium through diet. In addition, at the end of pregnancy, the demand for minerals derived from the mother for fetus bone solidification is greater [28]. Thus, the possible lower intake of calcium combined with an increase in demand of these minerals could produce a greater decrease in both minerals. However, future experiments

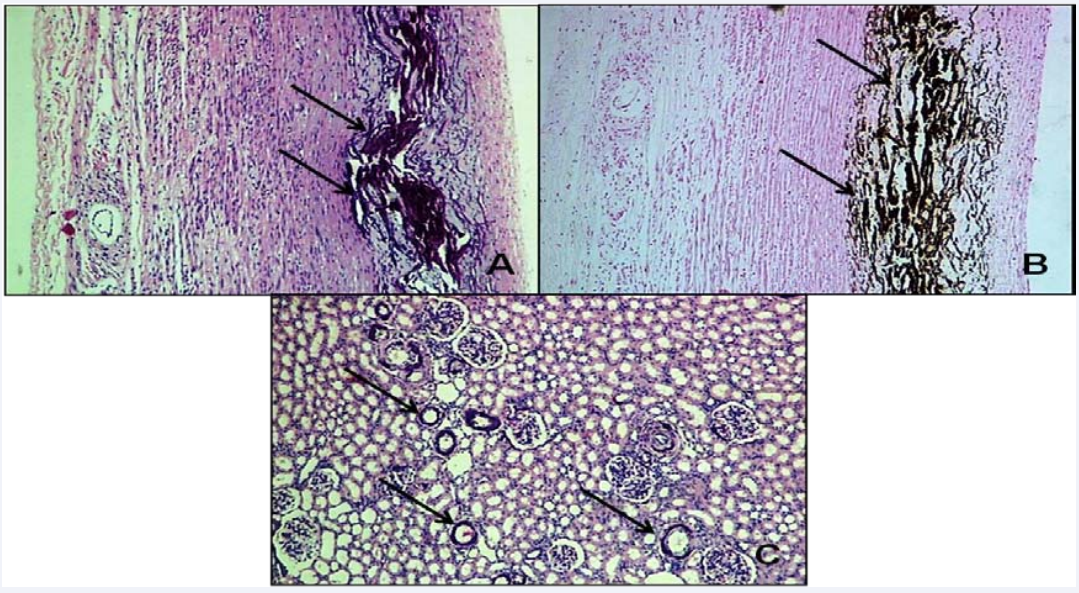

Figure 2 Light photomicrograph of young male goats treated with 200 mg/kg body weight daily of *S. malacoxylun*, dry-air leaves for 28 days. Aorta showing alteration in elastic laminae of tunica intima (A; arrow) and deposition of phosphate and carbonate (B; arrow). Kidney showing matrix alteration in the basal membrane of convoluted tubules and glomerulus (C arrow). Magnification: X 4, (A and C HE; B von Kossa stain).

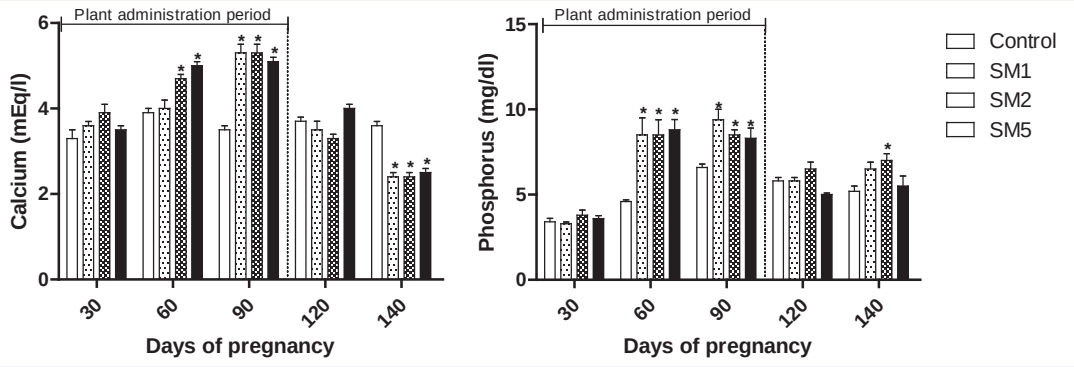

Figure 3 Serum level of calcium and phosphorus of pregnant goats allocated in 4 treatment groups: one control and three experimental SM1, SM2 and SM5 that received, respectively 12.5, 25 and 50 mg/kg body weight daily of *S. malacoxylun*, dry-air leaves from the 30th day of gestation to the 90th (*p<0.05; compared to control).

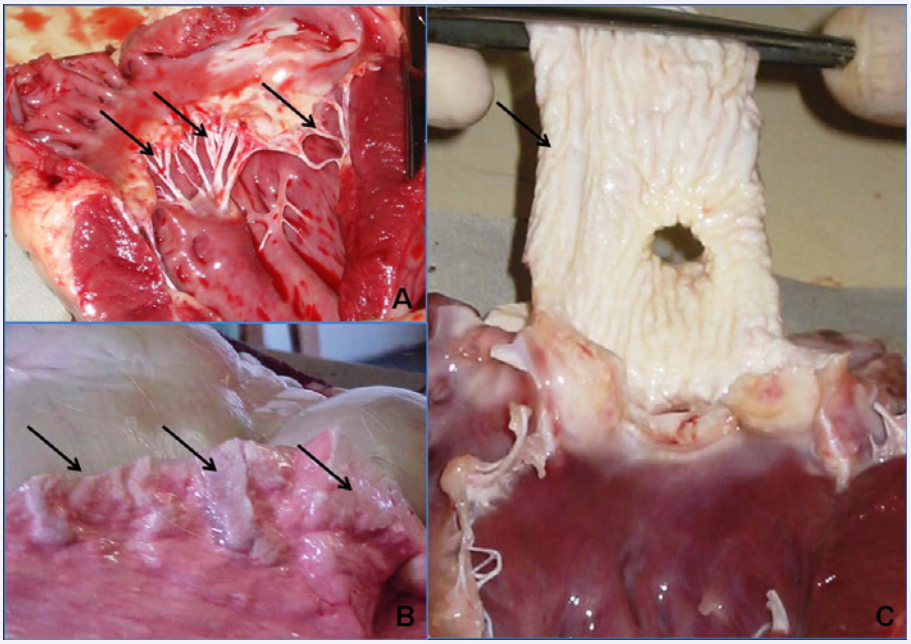

Figure 4 Photography of organs from female goat treated with 50 mg/kg body weight daily of *S. malacoxylun*, dry-air leaves from the 30th day of gestation to the 90th euthanized after delivery. Heart showing calcification of cardiac valves (A; arrow). Calcified pulmonary nodules (B; arrow). Calcified aorta (C; arrow).

will be conducted to better verify this hypothesis or to determine whether there is an impairment in the hormonal balance of calcitonin and PTH in pregnant goats exposed to *S. malacoxylon*.

Ultrasonographic evaluation at 35, 49, and 63 GD demonstrated no significant changes in FM, HR or in the morphometric measurements (CRL, TD, AD, and BPD) of the fetuses in any group studied (data not shown).

At necroscopic evaluation, it was possible to observe alterations in animals treated with *S. malacoxylon*. The heart and lungs were the most affected organs, showing intense calcification of the cardiac valves, cardiac muscle, aorta, pulmonary artery and calcified pulmonary nodules in a dose-dependent manner (Figure 4). Histopathological evaluation revealed alterations in all goats treated with *S. malacoxylon*. The histopathologic changes were dose related; thus, the lesions were more conspicuous in females treated with a larger amount of *S. malacoxylon*. Female goats had identical histopathological changes as growing male goats from trial 1, including alteration in elastic laminae of the tunica intima of the aorta and matrix alteration in the basal membrane of convoluted tubules and glomerulus (Figure 2).

Kids from the SM2 and SM5 groups also showed similar yet milder alterations in soft tissue similar to those of their mothers. Fetuses from goats fed 50.0 mg/kg BW of *S. malacoxylon* during gestation showed lower birth weight (Table 2, p>0.05). Previous work indicates placental transfer of 1.25 $(OH)_2D_3$ in sheep [29] and in humans [30]. Thus, we can assume that the effects observed here in newborn goats are the result of the direct action of calcinogenic glycoside that passed through the placenta.

Table 2: Reproductive parameters of pregnant female goats allocated in 4 treatment groups: one control and three experimental (SM1, SM2 and SM5) that received, respectively 12.5, 25 and 50 mg/kg body weight daily of *S. malacoxylun*, dry-air leaves from the 30th day of gestation to the 90th and the birth weight of their kids.

Parameters	Control	*S. malacoxylon* (mg/kg body weight)		
		12.5	25.0	50.0
Body weight gain (kg) during pregnancy[a]	17.4 ± 2.5(5)[b]	14.2 ± 1.4(5)	5.8 ± 1.0(5)*	5.87 ± 2.6(5)*
Length of gestation (days)[a]	150.1 ± 0.3	149.9 0.2	149.3 ± 1.8	150.3 0.7
Mortality of pregnant dams	0	0	1	0
Live kids	7	8	4	6
Male	3(43%)	5(62%)	1(25%)	3(50%)
Female	4(57%)	3(38%)	3(75%)	3(50%)
Twins	2(40%)	3(60%)	0(0%)	1(20%)
Kids body weight at birth[a]	3.5 ± 0.17	3.2 0.18	3.6 ± 0.29	2.8 0.23*
Fetal deaths/ Stillbirths/ Aborted fetuses	0	0	0	0

[a] Mean ± S.E.M.
[b] Number of dams.
*$p \leq 0.05$ compared with controls

CONCLUSION

Goats are a good animal model for studying the effects of *S. malacoxylon* in ruminants. The present data strongly support that, similar to rats [17] and rabbits [16], the toxic components of *S. malacoxylon* can affect both dams and fetuses from ruminants and that this form of intoxication may account for the heavy losses in the cattle industry.

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