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The Safety of *Viscum album* l. in a Murine Model: A Reproductive Toxicity Study

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Research Article

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ABSTRACT

Background: The use of antineoplastic drugs in pregnancy poses a huge safety challenge because of the risk of teratogenicity, mutagenicity and carcinogenicity. The rational use of the herbal medicine *Viscum Album* (VA) has shown promising results in several tumor lines as part of the strategy of immunotherapy and anti-angiogenesis in integrative oncology. The safety of intrauterine fetal exposure to VA is however unknown and can be further evidence for therapeutic doses in pregnant cancer patients.

Methods: 47 pregnant Wistar rats and 399 foetuses bred following exposure to VA were investigated. The rats were randomized into five groups. Control Group (CG) received no treatment and the Stress Group (SG) received daily vehicle subcutaneous injections alone in the same volume as the treatment groups from day zero until the twentieth day of pregnancy. There were three treatments groups: Therapeutic Dose Group (TG) received the Usual Therapeutic Dose (UTD) of 0.013 mg/kg BW; High Dose Group (HG) received 12.5 mg/kg BW and Very High dose Group (VHG) received 25 mg/kg BW, daily.

Keyword: *Viscum album*; Mistletoe; Reproductive toxicology; Cancer; integrative medicine; Phytotherapy; Traditional and complementary medicine; Safety; Pregnancy

Results: Weight gain was greater in the rats, placentas and fetuses in the TG and HG compared to SG. Histology of the placentas showed a greater inflammatory process in HG and VHG.

Conclusion: Since no cases of abortion, embryo toxicity, natimortality or teratogenicity were found in the fetuses and histology detected no lesions in the tissues evaluated, it is reasonable to conclude that this drug is safe for use in pregnant rats.

ABBREVIATIONS

CG: Control Group; GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor; HG: High dose Group; LD50: Median Lethal dose; ML: Mistletoe Lectins; QoL: Quality of Life; SG: Stress Group; TG: Therapeutic dose Group; VA: *Viscum Album* L; VAA: *Viscum Album* Agglutinins; VAE: *Viscum Album* Extract; VHG: Very High dose Group

INTRODUCTION

Cancer is the leading cause of death in women of reproductive age. During the last decades the incidence of cancer is increasing dramatically, with an incidence of 1 in 1,000 pregnancies and appears to be associated with the postponement of pregnancy and an early manifestation of cancer [1-5]. The incidence of pregnancy-associated breast cancer is increasing as women are delaying childbirth until later [6-9]. Recent data shows incidence of breast cancer during pregnancy as 1 in 3,000 to 10,000 birth deliveries, positioning these diagnosis as common as cervical uterine cancer [6-9]. Chemotherapy remains the cornerstone treatment for cancer. Although the drugs used in chemotherapy present a considerable carcinogenic risk to the pregnant woman as well as a teratogenicity risk to the fetus and a possibility of mutagenic effects on future generations, the extent of placental transfer varies considerably [4,10]. Considering the risks of toxicity in pregnancy, the search for new models of care based on whole medical systems, natural products and non-pharmacological therapeutic interventions has been increasingly addressed within the concept of integrative oncology to improve the quality of life and survival of these patients by integrating conventional therapy [11,12]. The search strategy for medicinal plants has resulted in the development and production of some anti-cancer agents widely used in oncology practice such as *Catharanthus roseus* (vincristine, vinblastine and vinorelbine), *Taxus brevifolia* (taxol and docetaxel), *Podophyllum peltatum* (etoposide and teniposide) and *Camptotheca acuminata* (topotecan) [13].

Of the integrative and complementary therapies used in oncology, extracts from the white-berry mistletoe (*Viscum album* L.), a hemi-parasitic plant belonging to the Santalaceae family, hemi parasites on a wide range of host trees in Europe, Asia and North Africa, have increasingly used in clinical practice [11,14-26].

The first meta-analysis performed in 1990 demonstrated the significant advantages of this drug in terms of overall survival and disease-free survival rates in cases of breast, lung, colorectal, stomach, uterine and ovarian tumors, and with respect to its efficacy in liver metastasis and its analgesic effect, which is mediated by endorphins [27,28]. Four years later, a second meta-analysis reported statistical significance only with respect to breast, lung and colorectal tumors [29-32]. Later, a large prospective study evaluated 10,226 patients and concluded that *Viscum Album* (VA) increased patients' survival time by 40% ($p<0.001$). The efficacy reported in that study corroborated the results of the meta-analysis performed in 1994 and once again included the uterus and stomach as sites that

could be beneficially treated with the drug [33]. A multicenter, retrospective cohort study conducted with 700 patients showed that a standard VAE represents a safe and effective co-adjuvant therapy for use following surgery for a primary breast tumor. This therapy results in a 4-6-fold reduction in side effects, consequently improving patients' well-being [34]. A systematic review of controlled clinical studies evaluating the effect of a VAE on the Quality of Life (QoL) of cancer patients showed that it was well tolerated. Moreover, it appears to improve QoL by reducing the incidence of the side effects experienced with conventional therapies (chemotherapy, radiotherapy). This has been demonstrated both in experimental trials and in routine daily use [35].

In a multicenter observational study was carried out in the Network Oncology in Germany, in the period between July 2003 and June 2013, with 2,805 patients received VA therapy (all forms of administration and 478 patients *via* I.V. infusion (10.2% of all cancer patients and 16.4% of VA patients). Lung cancer (23% of all) followed by pancreatic (18%), colorectal (17%), and breast (17%) cancer. In addition to VA, 77.5% patients received chemotherapy, 14.3% received immunotherapy, 13.1% hormonal therapy, 11.6% bisphosphonates and 6.3% signal transduction inhibitors, 78.3% had surgery and 34.1% radiation therapy [24]. A systematic review and meta-analysis on the survival of cancer patients treated with VA including eighty-two controlled studies indicate that adjuvant treatment can be associated with a better survival with most pronounced effects in cervical and less pronounced effects in lung cancer [36].

A systematic review and meta-analysis provide evidence that the *Viscum album* extracts produce a significant, medium-sized effect on QoL in cancer patients [37].

VA Extracts (VAE) is composed of a complex multi-component mixture with anticarcinogenic effects. The extracts contain various biologically active substances such as glycoproteins (lectins and VA chitin-binding agglutinin-VisalbCBA), polypeptides (viscotoxins), polysaccharides (arabinogalactans), thiols (glutathione), flavonoids (quercetin derivates) and triterpenes (oleanolic acid, ursolic acid and betulinic acid) [17,38-45]. The principal active components are the three mistletoe lectins (ML I, II, III), the isoforms of viscotoxins (A1-3, B, C1, 1-PS, U-PS) and the polysaccharide fractions [17,43,46-51].

Surface glycoconjugates of normal and transformed blood cells are commonly characterized by plant lectins to infer physiological significance of protein-carbohydrate interactions on cancer cells. When mannose- and galactose-binding lectins from several plants and from human serum/placenta were compared, binding of *Viscum Album Agglutinin* (VAA) to peripheral blood T-helper cells was found to be significantly higher [52]. In addition to its role in the inductions of apoptosis and immune modulation, some *in vivo* studies have highlighted an anti-angiogenic effect on endothelial cells. Comparing 24 different plant lectins to characterize glycoconjugate expression during the development of 13 to 21-day-old rat embryos, the affinity of VAA increased as the endothelial cells matured [53-57]. The median lethal dose (LD50) of VAE in rats is 378 mg/kg of body weight. The stimulation of immune system in Wistar rats has been established as 1.0 mg of ML^{-1}/kg as daily dose [58].

The therapeutic dose of VAE to induce cytotoxicity in human neoplastic cells (Iscador® Q 10 mg/mL) is 0.143 mg/kg BW or 54 mg of lectins/kg BW, corresponding to 0.05% and 0.26% of the LD50 for rats, respectively [21]. Safety with this dose is outstanding. Cytogenetic studies conducted with VA *in vitro* have reported negative effects with respect to mutagenicity for amniotic fluid cells, which serves as further evidence of the reliability of this drug [59–62]. Despite some authors' recommendation not to use VAE during pregnancy, there is no scientific evidence of teratogenicity and/or reproductive toxicity with VA [63]. Recently, some preclinical investigations showed that VAE is clearly non-genotoxic and exerts no relevant toxic effects on reproduction *in vivo* [64–66]. The objective of the present

study was to gather further evidence of the safety of VAE in pregnancy by evaluating its side effects on pregnant female albino rats and their fetuses.

MATERIALS AND METHODS

Female Wistar rats (Rattus norvegicus albinus) of the EPM-1 variant, weighing approximately 215 g each, were obtained from the Center for the Development of Experimental Models, Federal University of São Paulo (UNIFESP). The study was approved by the local Animal Care Committee and all experiments were performed according to the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care in Science and the Guide for the Care and Use of Laboratory Animals [67,68].

Forty-seven animal and the 399 fetuses resulting from their pregnancies were evaluated in this study. The animals were given free access to food and water. After mating, pregnancy was diagnosed from the detection of spermatozoids in vaginal smears, thus defining day zero [69]. The pregnant rats were randomly divided into five groups. The Control Group (CG) received no medication and the Stress Group (SG) received the drug vehicle (1 mL of saline solution (0.9% NaCl) administered Subcutaneously (SC) into the posterior cervical region. The experimental groups, consisting of Therapeutic dose Group (TG), the High Dose Group (HG) and the Very High dose Group (VHG), received *Viscum album* Qu 5 mg special (Iscador AG, Arlesheim, Switzerland) diluted in 1 mL of vehicle at the doses of 0.013, 12.5 and 25 mg/kg BW/day, respectively, also administered SC into the posterior cervical region. The drug was given from the 1st to the 20th days of pregnancy and corresponded to 0.98, 937 and 1875 mg of lectin II/kg BW, respectively, or 1, 961 and 1,923 times the usual therapeutic dose, respectively. The very high dose correspond to 6.6% of the LD50.

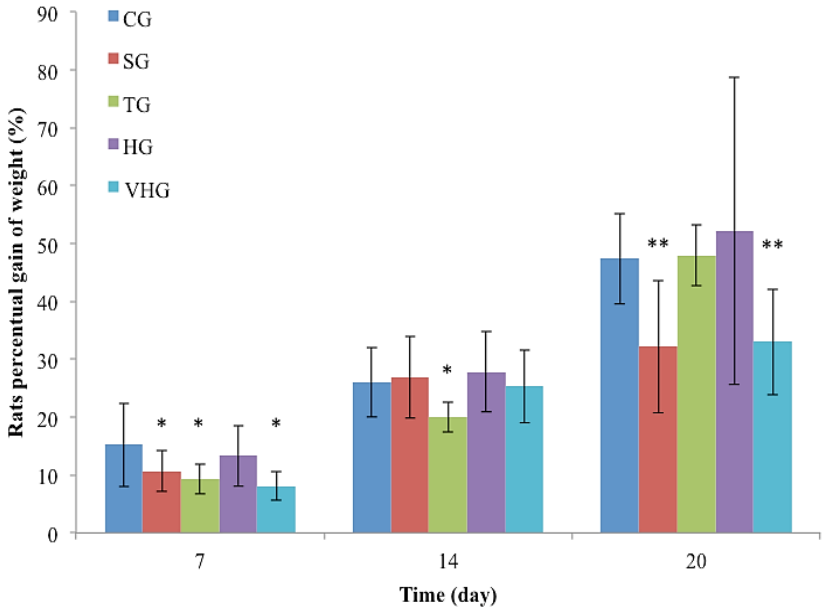
The rats were weighed on the 7th, 14th and 20th days of pregnancy and the increase in body weight was recorded as a percentage. When the pregnancies reached full term, all the rats were anesthetized using mixture of zylazine (20 mg/kg) and ketamine (100 mg/kg), administered intraperitoneally, after which laparotomy and hysterectomy were performed. The following parameters were recorded: ovum implantations, ovum reabsorptions, number of live and dead fetuses, fetal and placental weigh and morphologies. The heart and thymus from 50 fetuses (10 from each group) were weighed and submitted to histology. After the thymus was extracted, an in situ mesoscopic study was carried out on the heart. The great vessels and the cardiac septa, valves and chambers were evaluated in accordance with blood flow. Of the 399 fetuses, 384 were examined using the technique first described by Wilson and modified by us [70]. In brief, a median sagittal section was made along the body of the fetuses extending from the oral cavity to the tail, which permitted detailed evaluation of the entire central nervous system without interruption, beginning at the brainstem and extending up to the caudal extremity of the spinal cord [71]. The internal structures located in a linear cranial-caudal sequence, were also inspected with respect to their syntopy: trachea, esophagus, lungs, pleura, pericardia, diaphragm, liver, stomach, small and large bowels, kidneys, adrenal gland, bladder, gonads and great vessels. Sections of ten (two per group) of the five fetal organs (liver, kidneys, heart, brain and thymus) were stained with hematoxylin and eosin for histologic evaluation. In addition, five placentas were studies (one per group). A set of ten thymuses (two per group) was submitted to immunohistochemistry to evaluate the expression of CD57 (Natural Killer (NK) cell marker) using light microscopy, with 400 × magnification.

Data were expressed as mean ± Standard Deviation (SD). Anova and Kruskal-Wallis multiple comparison tests were used for the statistical analyses. The chi-square test was used to compare macroscopic alterations between groups. Significance was considered when $p<0.05$.

RESULTS

No maternal deaths occurred in any animal group. As there was a significant difference between the groups with respect to the weight of the rats (p=0.011), the percentage weight increase from baseline was calculated at the 7th, 14th and 20th days and compared between the groups (Figure 1).

Figure 1. Weight gain on the 7th, 14th and 20th day of pregnancy in rats according to the study group. Note: (■) CG; (■) SG; (■) TG; (■) HG; (■) VHG



The graph in Figure 1 shows the mean percentage weight gain in the rats at the 7th, 14th and 20th days for the animals in the different study groups.

As expected, significant differences were found when weight gain at the 7th, 14th and 20th days was compared with baseline weight (p<0.05), considering each period individually, weight gain was greater in the CG and in HG compared to the other groups (Table 1).

Table 1. Statistics of rats' weight at the 7th, 14th and 20th days according to the study group.

	Δ% day 7	Δ% day 14	Δ% day 20
Group	Mean ± SD	Mean ± SD	Mean ± SD
	Median	Median	Median
	Min./Max.	Min./Max.	Min./Max.
CG n=10	15.2 ± 7.2	26.0 ± 6.0	47.3 ± 7.8
	14.5	24.3	46.4
	5.7/33.2	18.7/36.6	38.3/61.1
SG n=9	10.7 ± 3.5	26.8 ± 7.0	32.1 ± 11.4
	10.2	26.8	32.7
	5.3/15.5	16.4/37.2	17.1/46.7
TG n=11	9.3 ± 2.6	20.0 ± 2.6	47.9 ± 5.3
	9.1	19.9	48.5
	4.4/13.6	16.0/25.0	36.8/55.1

HG n=9	13.3 ± 5.2	27.8 ± 6.9	52.1 ± 26.5
	11.6	27.8	53.4
	4.5/21.3	14.9/37.0	6.8/109.0
VHG n=8	8.1 ± 2.5	25.3 ± 6.3	33.0 ± 9.1
	8.8	26.4	33.2
	3.4/10.3	15.7/34.7	12.6/42.6
Note: Δ% day 7: p=0.011* CG ≠ SG; CG ≠ TG; CG ≠ VHG; HG ≠ VHG; Δ% day 14: p=0.037* CG ≠ TG; SG ≠ TG; TG ≠ HG; Δ% day 20: p=0.008* CG ≠ SG; CG ≠ VHG; SG ≠ TG; SG ≠ HG; TG ≠ VHG; HG ≠ VHG			

Nevertheless, over the 14-day period, the increase in weight was greater in the SG, a gain that was similar to that recorded in the CG and HG groups. Over the 20 days of pregnancy, the weight gain was smallest in the SG and VHG groups, with no statistically significant differences between these groups.

Of the 399 fetuses born to the rats in this study, 96.24% (n=384) were examined using the modified Wilson technique. Only one abnormality, a case of hydrops fetalis, was found in the TG. Since this case represents 0.26% of the sample, being the only fetal abnormality found in the study, it is impossible to establish any correlation with the drug tested. No miscarriages or reabsorptions occurred. A statistically significant difference was found when the number of fetuses per pregnancy was compared between the groups (p=0.018). The multiple comparison tests showed that the greatest number of fetuses was found in the CG, with a mean of 11.2 fetuses per pregnancy compared to 6.8 in the SG and 6.5 in the VHG, these differences being statistically significant. There was no statistically significant difference between the SG and VHG groups, which would appear to refute any correlation with the drug (Table 1). With respect to fetal weight, the present data show a statistically significant difference between the groups (p<0.001). The lowest mean fetal weight was found in the HG (Table 2).

Table 2. Mean fetal weight (g), corrected in accordance with the mean number of fetuses per pregnancy.

Mean fetal weight	CG	SG	TG	HG	VHG
Mean weight (g)	4.20	4.08	4.17	3.63	4.14
Mean number of fetuses per pregnancy	11.20	6.80	8.70	8.70	6.50
Corrected fetal weight (g)	4.70	3.08	3.30	3.51	3.36

However, it is known that an inverse correlation may exist between the number of fetuses per pregnancy and fetal weight. Therefore, mean fetal weight was corrected by calculating the mean number of fetuses per pregnancy. The highest mean corrected fetal weight was in the CG (4.7 g), followed by the SG and TG groups, with 3.08 g and 3.30 g respectively.

Analysis of placental weight showed a statistically significant difference between the groups (p<0.001) (Table 3). The multiple comparison tests showed the highest mean placental weight to be in the CG followed by the TG. A

statistically significant difference was found between these two groups and the HG, the group with the lowest mean placental weight. Table 3 shows the mean placental weight recorded in the different groups following correction using the same principle that was applied to fetal weight. Following correction, the mean placental weight of 0.60 g found in the HG is higher than that found for the TG and VHG groups, which are similar, with 0.55 and 0.54 g respectively. Placental weight remained lowest in the SG (0.49 g) (Table 3).

Table 3. Mean placental weight (g), corrected in accordance with the mean number of fetuses per pregnancy.

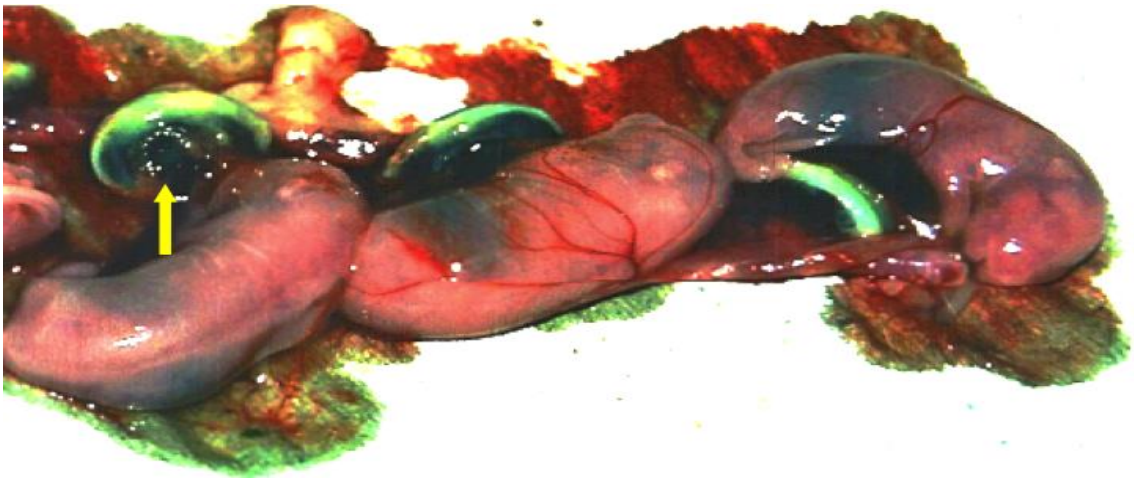
Mean placental weight	CG	SG	TG	HG	VHG
Mean weight (g)	0.79	0.66	0.69	0.62	0.66
Mean number of fetuses per pregnancy	11.20	6.80	8.70	8.70	6.50
Corrected placental weight (g)	0.89	0.49	0.55	0.60	0.54

No abnormalities or malformations were found in any of the 50 hearts evaluated by dissection. Light microscopy carried out on the five fetal organs (liver, kidney, heart, brain and thymus) failed to reveal any morphological alterations. The weight of the thymus, evaluated in 10 fetuses from each group, showed no statistically significant differences between the groups (p=0.397).

There was an increase of 23.1% in the number of CD57-positive lymphocytes (NK) in the TG compared to the other groups: 7.1% (VHG), 10.7% (SG), 11.1% (CG) and 12.5% (HG). However, the differences between the groups were not statistically significant (p=0.350).

External examination of the discoid placenta showed a macroscopic pattern of fibrin at the edges that was restricted to the eight animals in the VHG (Figure 2).

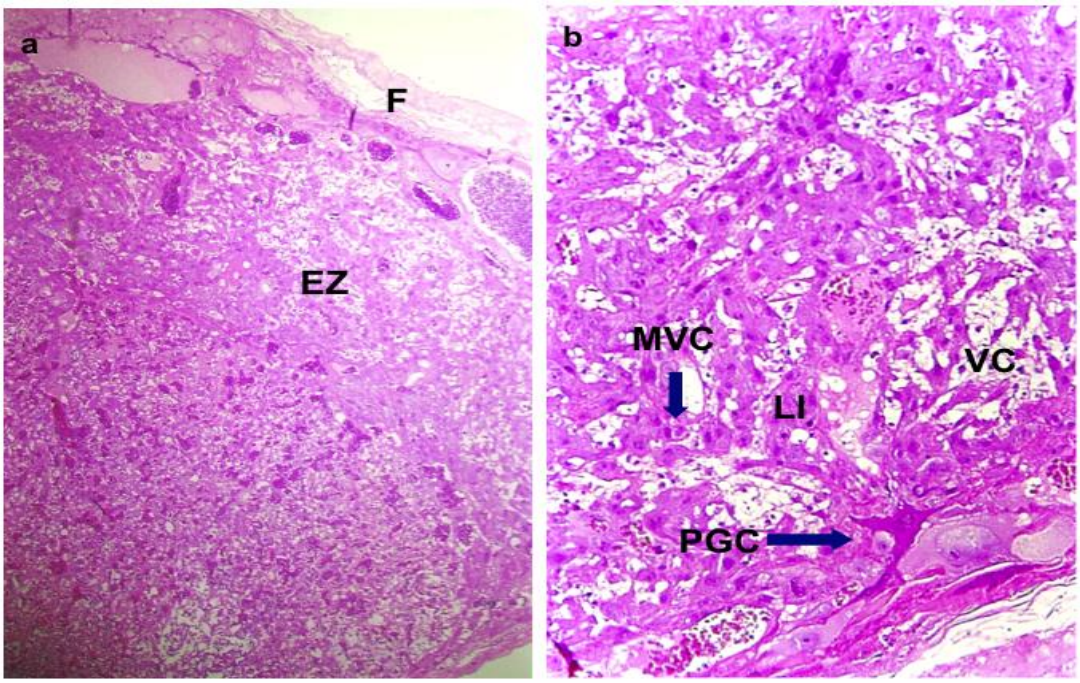
Figure 2. Image of three fetuses from the Very High Dose Group (VHG). **Note:** the discoid placentas and the altered color of the edges of the placenta (arrow)



These modifications suggest the presence of a more intense inflammatory event in this group. Taking into consideration the known histology of the rat placenta, microscopic evaluation revealed seven abnormalities in the HG and VHG groups on the 20th day of pregnancy. These abnormalities consisted of: 1) thickening of the external zone; 2) an increase in the uterine sinus; 3) leukocyte infiltration; 4) large vacuoles; 5) presence of fibrin in the external surface; 6) predominance of a mononuclear morphology in vesicular cells; and 7) fewer plasmodia. The

first are associated with inflammation, while the final two concern placental immaturity. While the first three alterations were more severe in the VHG, the final three occurred exclusively in this group (Figure 3).

Figure 3. Histology of placenta in the VHG. Note the presence of large Vacuole Cells (VC), plasmodia giant cells (PGC), Mononuclear Vesicular Cells (MVC) and Leukocyte Infiltration (LI) of the External Zone (EZ), covered by Fibrin (F). Hematoxylin-eosin staining. (Magnification 40X in photography a and 100X in photography b).



DISCUSSION

The poorest weight gain at the end of pregnancy in the SG clearly highlights the stimulus effect. Although all the groups treated with the drug received the same stress stimulus, the groups treated with therapeutic doses and high doses presented a compensatory behavior at the end of the pregnancy in relation to weight gain, achieving the same increase in weight as that recorded in the CG. This effect can be explained by the inflammatory Th1 pattern shared by both groups and which would induce an overall increased blood flow to organs and tissues, particularly to the placenta and fetuses. On the other hand, in the animals in the group receiving the maximum dose (VHG), whose weight did not differ from that of the animals in the SG, no such compensatory benefits was found, most likely due to cytotoxicity and the anti-angiogenic effect of VA.

To evaluate fetal and placental weight, the two variables-the effect of the drug and the mean number of fetuses per pregnancy-had to be separated. As shown in Tables 1 and 2, these results are coherent. From these data, it is feasible to infer that the lower mean placental and fetal weight found in the HG were a consequence of the greater mean number of fetuses per pregnancy in this group.

Despite data form literature showing a two-fold increase in the weight of the thymus due to the proliferation of cortical thymocytes in adult rats, the present data failed to find any differences in the weight of prenatal thymus [59,72,73]. These findings suggest that during the prenatal development of the thymus, the effect of VA on the proliferative response of thymocytes was not significant. Nevertheless, the two-fold increase in the population of NK

cells with the therapeutic dose (23.1% compared to 7.1-12.5% in the other groups) agrees with data in the literature [59,72,73]. The lack of statistically significance results was due to the few CD57-positive cells, which may be related to the reduced differentiation rate in this phase of development.

Histologic examination of the five fetal organs failed to show any signs of toxicity at any of the drug doses tested. Histology of the placenta showed that the inflammation provoked by mistletoe lectin may be responsible for the abnormalities found. The reported binding of VAA to human serum/placenta and its affinity to amniotic fluid cells partially explains the present findings [52,59,64]. The presence of large vacuolated cells, particularly in the VHG, indicates cytotoxicity associated with the inflammatory process in a dose-dependent manner. The mononuclear rather than binuclear morphology of the vesicular cells, together with the reduced number of giant plasmodia at the end of pregnancy, suggests that in the VHG the drug provoked a slight delay in placental maturity mediated by apoptosis and the anti-angiogenic effect of the MLs.

Previous preclinical studies reporting a lack of mutagenic and carcinogenic risks, together with the *in vivo* results of the present study, suggest that it may be safe to use VA in therapeutic doses in pregnant cancer patients.

CONCLUSION

The local inflammatory process in the placentas of Wistar rats subjected to daily subcutaneous high doses (961 times and 1,923 times the usual therapeutic dose) of *Viscum album* Qu 5 mg special (VA) point to a pattern of dose-dependent affinity with the placenta. The absence of signs of teratogenia, macroscopic embryotoxia, or any microscopic changes in fetal liver, kidney, heart, brain and thymus tissues in all groups, in addition to the finding of weight gain in pregnant rats, fetuses and placentas after daily use of therapeutic and high therapeutic doses compared com stress group, allows VA to be considered a safe drug in Wistar rats even when administered at high doses during pregnancy. The finding of increased NK lymphocyte numbers in the fetal thymus at the usual therapeutic dose suggests a potential immunomodulatory effect in fetal medicine.

DECLARATIONS

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors confirm that there are no conflicts of interest associated with this publication.

Funding

Not applicable.

Author's contributions

LKJ, MUN, RG and MCSPC were responsible for the overall study design and concept; RG was responsible for writing the manuscript under the supervision of JKH and MUN; MMSI for supervision in the macroscopic analysis of fetal hearts; MJS for supervision in the histological analysis of fetal tissues; MMSI, MJS, LKJ and MUN for the

interpretation and discussion of results; JVCB for critical revision of the article. All authors read, reviewed and contributed to the final manuscript.

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