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In-Vitro Growth Kinetics of Mesenchymal Stem Cells in Cytotoxicity Tests Using Low-Diluted *Viscum Album*

Ana Catarina Viana Valle[1,2]   Hilana dos Santos Sena Brunel[3]   Bruno Stéfano Lima Dallago[4]
Lucas Santana Rodrigues[5]   Patrícia Furtado Malard[2,5]   Rosiane Andrade da Costa[2]   Rafael Rossetto[2]
Rosângela Vieira de Andrade[2] iD

[1] Doctor Izao Soares Institute, Sao Paulo, Brazil
[2] Graduate Program in Genomic Sciences and Biotechnology, Catholic University of Brasilia, Brazil
[3] BioInnova, Brazil
[4] Brasilia University, Brazil
[5] BioCell Cell Therapy, Brazil

Address for correspondence Rosangela Vieira de Andrade, PhD, Graduate Program in Genomic Sciences and Biotechnology, Catholic University of Brasilia, SGAN 916, Brasília, DF 70790-160, Brazil (e-mail: rosangelavand@gmail.com).

Abstract

Introduction The use of mesenchymal stem cells (MSC) in cytotoxicity tests is an *in-vitro* alternative model for predicting initial doses. Homeopathic medicines may stimulate the immune system to combat a pathology effectively and have been used for over two centuries. *Viscum album* (VA) extracts are widely used in the treatment of cancer, due to their immunomodulatory, cytotoxic and pro-apoptotic properties.

Objective This study aimed to evaluate the *in-vitro* growth kinetics of canine MSC in relation to cytotoxicity, cell differentiation and expression of pluripotentiality markers, using a VA preparation at the D1D2 (1×10^{-1}, 1×10^{-2} potency (VAD1D2).

Methods MSC were obtained from adipose tissue sampled from a healthy dog that was undergoing an elective veterinary procedure and with its owner's permission. The experiments were performed in three groups: MSC treated with VAD1D2 or diluent or untreated (control). The cytotoxicity was evaluated by MTT assay. The differentiation was induced in three lineages, and apoptotic cell labeling was performed by an Annexin-V test.

Results At the concentration of $10\,\mu L/mL$ of VA, the number of cells after *in-vitro* culture was maintained when compared with the control (untreated) group. A significant and gradual decrease in cell viability was recorded as VA concentrations increased. The apoptosis analysis showed that VA at $20\,\mu L/mL$ presented absolute percentages of initial apoptosis twice as high as at $10\,\mu L/mL$, which was similar to the control (untreated group).

Conclusion The results suggest that the use of efficient methods to assess the *in-vitro* cytotoxicity of VA-based homeopathic medicines using MSC lineages may predict the potential action at different concentrations. These findings demonstrated that VAD1D2 interferes with canine MSC growth kinetics.

Keywords
► apoptosis
► cell viability
► cell differentiation
► complementary therapy

Introduction

Homeopathic medicines may stimulate the body's immune system to combat a particular pathology. Though the mechanism of action remains unknown, some studies have demonstrated that these preparations can retain biological properties from their starting vegetal extracts.[1–3] The use of *Viscum album* (VA; mistletoe) extract in the treatment of cancer diseases has been reported over the years, due to its immunomodulatory, cytotoxic, pro-apoptotic, anti-angiogenic, and DNA-stabilizing properties.[4,5]

The mechanism of action of VA varies according to its composition and has been of growing interest since its cytotoxic and immunomodulatory properties were first documented.[6,7] Its metabolic activity is mediated by biologically active compounds, especially viscotoxins and lectins,[8–11] which exert a direct cytotoxic effect on tumor cells,[12] and by triterpenes, which enhance the activity of natural killer cells by selectively activating their anti-tumor capacity and apoptotic properties.[13–15] Moreover, its bioactive compounds induce anti-inflammatory activity by the selective inhibition of cyclooxygenase-2 (COX-2), which is transcriptionally activated in response to various pro-inflammatory cytokines,[16] showing a synergistic effect on the anti-angiogenic process, which is fundamental in tumor regression and metastasis inhibition.[17]

The biophysical properties of homeopathically diluted preparations of VA remain active. Such preparations create an upper layer of nanoparticles that retain the energy of matter after several dilution steps, a process known as dynamization.[18] Despite the controversy on this topic, the use of complementary or alternative medicines in the treatment of cancer has increased in recent years,[3,19–21] and studies have reported clinical cases that support the beneficial effects of VA as a complementary cancer therapy.[22] In this context, *in-vitro* studies can contribute to excluding any placebo effect of homeopathic medicines.[23]

Despite the intense use of complementary therapies in recent decades, the intrinsic cellular mechanisms involving the anti-tumor action of VA are still poorly understood.[24] The diversified preparations and different methods of application lead to a variability of therapeutic responses,[25] which may contribute to the poor elucidation of the cellular mechanisms of action caused by homeopathy. Furthermore, the intrinsic complexity of cancer itself and of the VA composition represent limitations for animal clinical trials. However, safety trials are urgently needed to reduce side effects, to validate the efficacy, and to understand the mechanism of action of this medicine more deeply.[26]

The definitive inclusion of mistletoe (VA) therapy is thus challenging due to the presence of few controlled evaluations of anti-tumor activity and cellular cytotoxicity tests. Therefore, this study aimed to evaluate the interaction between canine mesenchymal stem cells (MSC) and a preparation of VA at the D1D2 potency. This was performed by analyzing the cytotoxicity, apoptosis, cell differentiation, and expression of pluripotentiality markers of these cells when cultured in the presence of different concentrations of the VA preparation.

Methods

Product Tested

The VA at the D1D2 (1×10^{-1}, 1×10^{-2}) potency consists of the combination of equal parts of two potencies, based on the 'potency chords' concept, which suggests that the combination of two potencies could be more effective than the use of the isolated potencies.[27] VAD1D2 was obtained from the company Injectcenter (Ribeirão Preto, São Paulo, Brazil) and comprised 1.1 mL ampoules. The preparation of injectable VA D1D2 was performed according to the international compendium, the German Pharmacopoeia. Briefly, the basic dosage form Mother Tincture (MT) is taken as a starting point. Following the Hahnemannian decimal method, one part of the MT was added to nine parts of the inert ingredient (sterile isotonic solution), succussing 10 times and thus obtaining VA D1 (10^{-1}). Then, one part of the preparation VA D1 was added to nine parts of the inert ingredient, succussing 10 times, and obtaining VA D2 (10^{-2}). To obtain VAD1D2, one part of VA D1 was added to one part of VA D2. Each preparation was packaged in 1.1 mL ampoules, in a classified and certified area. The process is validated, thus guaranteeing the sterility and quality of the final product.[28]

Tissue Sample Collection, Isolation and Cultivation of MSC

The experiment that involved an animal in this study was approved by the Ethics Committee on Animal Use, Catholic University of Brasília (CEUA - UCB), Brazil (CEUA No. 002/1619)—(►**Supplementary file S1**, available online only). The adipose tissue samples were obtained from a healthy canine donor that was undergoing an elective castration procedure by a licensed veterinarian. A signed consent form was obtained from its owner in advance. Anesthesia was induced by intravenous propofol 6 mg/kg (Propovan, Cristália Produtos Químicos e Farmacêuticos Ltda., Itapira, Brazil) and sustained with inhalational isoflurane (Vetflurano, Virbac, São Paulo, Brazil). A small incision (5 cm) was made at the base of the animal's lumbar region, where a small amount of fat (20 g) was removed (►**Fig. 1A**). The procedure for collecting the stem cells took approximately 8 minutes and the skin was then sutured with 3–0 nylon thread, with two separate simple stitches, at the incision site. The stitches were removed after 7 days, and the castration stitches were removed after 10 days. The animal's post-operative period was uneventful, and it completed recovery in 1 week, with no apparent ill effects, since it had been submitted to elective castration and the cell collection procedure was secondary to that.

The dog was free of any infectious or parasitic symptoms and was submitted to hematological and biochemical tests to certify its health status, according to protocols previously described by De Francesco et al.[29] The animal was handled, and all the procedures were performed, in accordance with European Directive 2010/63/EU.

After collection, the adipose tissue was mechanically fragmented and submitted to enzymatic digestion with collagenase II (1 mg/mL) for 30 minutes. The cell solution
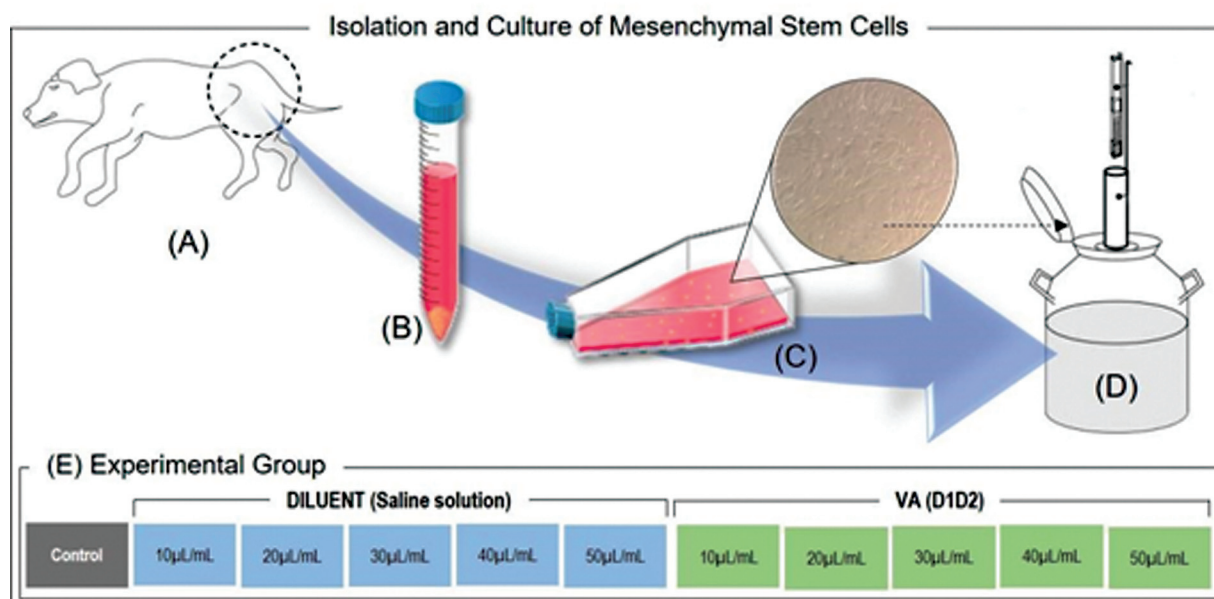
Fig. 1 Experimental organization chart of the isolation stage and cultivation of MSC. (**A**) Collection of adipose tissue fragments during veterinary anesthesia; (**B**) fragmentation and enzymatic digestion; (**C**) plating and cultivation; (**D**) MSC cryopreservation; (**E**) distribution of experimental groups for *in-vitro* cultivation. All procedures were conducted by a licensed veterinarian.

obtained was double filtered through a 100 μm and 40 μm mesh, and then centrifuged at 1,250 rpm for 3 minutes (►**Fig. 1B**). The cell pellet obtained was then washed twice in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum and 0.02% of amikacin, which was then defined as base MEDIUM.

The pellet was re-suspended, and cells were plated at a concentration of 2.5×10^3 cells/mL of medium on 25 cm^2 flasks and cultured *in vitro* at 37.5°C, 5% CO_2, and saturated humidity (►**Fig. 1C**). The MEDIUM was replaced after 24 hours, and subsequent changes were performed every 2 to 3 days, along with the evaluation of cell morphology and confluence state under an optical microscope. Upon reaching 70% confluence, the cells were dissociated using Trypsin-EDTA (Sigma-Aldrich) reagent and then expanded to the second passage (P2), cryopreserved in 0.25 straws (1×10^6 cells/straw) and stored in liquid nitrogen (►**Fig. 1D**). To evaluate cell morphology, MSC quantification and the cytotoxicity of VAD1D2, the MSC (P2) were cultured in base MEDIUM for 72 hours in the following experimental groups: control (no addition of saline or VA); diluent (addition of saline at concentrations 10, 20, 30, 40, and 50 μL/mL); and VA (with addition of VAD1D2 at concentrations of 10, 20, 30, 40, and 50 μL of VA/mL) (►**Fig. 1E**). The *in-vitro* culture base MEDIUM, exchange volume, and experimental conditions were similar to those previously cited.

MSC Characterization by Flow Cytometry Immuno-phenotyping

Canine MSC (P2) cultured in the absence (control group) or the presence (VA groups) of VA before and after cryopreservation were subjected to characterization by flow cytometry immuno-phenotyping with image quantification (ImageStream Mark II Amnis—Millipore, Seattle, Washington, United States).

Data were analyzed using the IDEAS software. For this purpose, MSC from each experimental group were individually incubated with fluorochrome-conjugated antibodies for 15 minutes at room temperature, protected from light. These MSC were used for multiplex analysis with cell membrane surface markers anti-CD29 (rat anti-human CD29-RD1), anti-CD44 (rat anti-equine CD44-FITC), and anti-CD105 (rat anti-human CD105-AF647). Cell undifferentiation markers were used for anti-SOX2 intranuclear proteins (primary SOX2 rabbit anti-human and DL488-conjugated rat anti-rabbit IgG) and anti-OCT3/4 (rat anti-human OCT3/4 and DL488-conjugated rabbit anti-rat IgG).

Multilineage Differentiation

MSC (P2) were cultured *in-vitro* in the base MEDIUM for 24 hours for homogenization and stabilization. They were then induced to differentiate into chondrogenic, osteogenic, and adipogenic lineages using commercial differentiation kits (A10064–01—StemPro, Gibco Life Technologies, United States), following the manufacturer's instructions. As a negative control, MSC were cultured in the base MEDIUM for 14 or 21 days according to the medium replacement regimen and *in-vitro* culture conditions.

(a) *Chondrogenic:* For chondrogenic differentiation, 1.3×10^6 cells/mL were plated and cultured in a 24-well plate containing 500 μL of Chondrogenesis Differentiation Medium. A 5 μL droplet of cell solution was added to the center of the well and allowed to settle and stabilize for 2 hours in an incubator, for micromass formation. After this procedure, 500 μL of differentiation medium was added, which was replaced every 3 to 4 days for 14 days. At the end of the culture period, the cells were fixed in 4% paraformaldehyde for 30 minutes, washed in

PBS, and stained with Alcian Blue for optical microscopy evaluation ($400\times$ magnification) to identify glycosaminoglycans present in the matrix of chondrogenic cells derived from the synthesis of proteoglycans by chondrocytes.

(b) *Osteogenic:* For osteogenic differentiation, 1.3×10^6 cells/mL were cultured in monolayers in 24-well plates containing 500 µL of MSC Growth Medium per well. After the cells reached 70% confluence, the medium was replaced by the Osteogenesis Differentiation Medium, and the cells were cultured for 21 days, replacing the medium every 3 to 4 days. At the end of the culture period, the cells were fixed in 4% formaldehyde for 30 minutes and subjected to Alizarin Red S staining (pH 4.2).

(c) *Adipogenic:* For adipogenic differentiation, 1.3×10^6 cells/mL were cultured in monolayers in 24-well plates using 500 µL of MSC Growth Medium per well. After the cells reached 70% sub-confluence, the medium was completely replaced by the Adipogenesis Differentiation Medium, and the cells were cultured *in-vitro* for 14 days, replacing the medium every 3 to 4 days. At the end of the culture period, the cells were fixed in 4% formaldehyde and stained with Oil Red for 20 minutes to evaluate the presence of lipid droplets.

MTT Assay

MSC were cultured in 96-well plates containing 5×10^4 cells/mL in 100 µL of base MEDIUM at 37.5°C, 5% CO_2, for 24 hours for cell stabilization and adhesion. This procedure was performed in two separate plates: half the plates would be used for the MTT assay and the other half for quantification. After this period, the base MEDIUM was replaced, and the evaluation factor, which contained the diluent and/or VA D1D2 at different concentrations, were added to each group. MSC from all experimental groups and one untreated group (control) were cultured for a further 48 hours. The culture medium was then removed from half of the plates, and 20 µL of the solution containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Catalog: Sigma N. M2128) at 0.5 mg/mL was added to each well and incubated for 4 hours at 37.5°C, protected from light. Subsequently, the supernatant was removed, 100 µL of DMSO was added to each well, homogenized, and absorbance then recorded at 570 nm on a microplate spectrophotometer (Molecular Devices, Sunnyvale, California, United States) for identification of viable cells. In the other half of the plates, after treatment of each cell group, the cells were harvested by trypsinization and quantified using a Neubauer chamber observed under microscopy, all performed by the same technician. The experiment was performed in triplicate and repeated three times.

Apoptotic Cell Labeling by Annexin-V

Apoptotic cell labeling was performed using the Annexin-V kit (Annexin/Alexa Fluor 488 and Propidium Iodide/PI—ThermoFischer Scientific). MSC from the culture groups, namely control, diluent 10 µL/mL, and VA 10 µL/mL and 20 µL/mL, in addition to previously cryopreserved MSC, were used for this assay. Cells were washed twice in 200 µL buffer followed by centrifugation at 1,500 rpm for 5 minutes. After centrifugation, the supernatant was dis-carded, the cells re-suspended in Annexin-V mix, and then incubated for 15 minutes, protected from light. The cells were re-washed and placed in 500 µL of PBS for immediate evaluation. Data were obtained using Amnis imaging flow cytometry (Millipore, Seattle, Washington, United States). The population of intact cells in early apoptosis, late apoptosis and necrosis was evaluated. The experiment was performed in triplicate.

Statistical Analysis

Statistical analyses of cell counts were performed using SAS software (v.9.4, Cary, North Carolina, United States) and based on a 5% significance level. Data from the cell viability test were subjected to Normality analysis using the Shapiro-Wilk test. They were then evaluated for differences between the experimental groups by Wilcoxon test. Data were subjected to regression analysis (PROC REG) for the relevant polynomial regressions. The results of the MTT assays were analyzed using GraphPad Prism 7.04 software, using the Tukey test for multiple comparisons.

Results

Morphology and Quantification of MSC

The effect of different VA concentrations on dynamized D1D2 and the effect of its diluent on MSC viability after 72 hours of *in-vitro* culture are shown in ►**Fig. 2**. The number of cells did not change after *in-vitro* culturing for 72 hours, regardless of the diluent concentration used, when compared with the control group. Moreover, it was observed that VA at 10 µL/mL did not influence the MSC proliferation pattern, resulting in the same values as in the control and the diluent used at the same concentration. However, a significant decrease in the number of MSC was observed after 72 hours of *in-vitro* culture with VA at 20 and 30 µL/mL concentration when compared with control and with diluent at the same concentrations. Suppression of cell proliferation was observed at VA concentrations of 40 and 50 µL/mL, resulting in the absence of MSC at the end of the *in-vitro* culture period. Similar MSC morphological characteristics over 24, 48, and 72 hours of *in-vitro* culture were observed between the control group (►**Fig. 3A**) and the group with 10 µL/mL of VA (►**Fig. 3B**).

MSC Characterization

(a) Immuno-phenotyping

Protein expression data for the MSC characterization were confirmed for all experimental groups by the positive expression of CD29, CD44, SOX2, and OCT3.4. In ►**Fig. 4**, the positive triple-expression analysis for CD44, CD29 and CD105 labeling for both the control group and the VA treatment provide confirmation that 96% and 98% of the MSC, respectively, retained their potential for undifferentiation. Furthermore, the MSC showed positive expression of SOX2 and OCT3/4, evidencing the pluripotentiality-regulating transcription factors in the control and VA-treated groups.
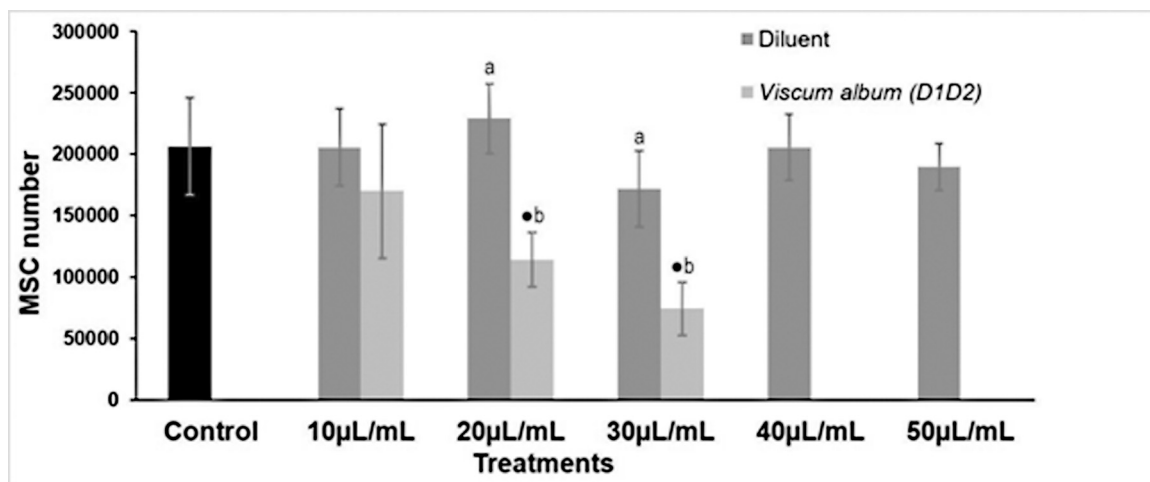
Fig. 2 MSC quantification after 72 hours of *in-vitro* culture in the control group, diluent group (addition of saline at concentrations of 10, 20, 30, 40, and 50 µL/mL) and VA group (addition of VA at 10, 20, 30, 40, and 50 µL/mL). Different letters indicate significant differences between treatments (VA and diluent). * Indicates significant differences in relation to the control group ($p < 0.05$).
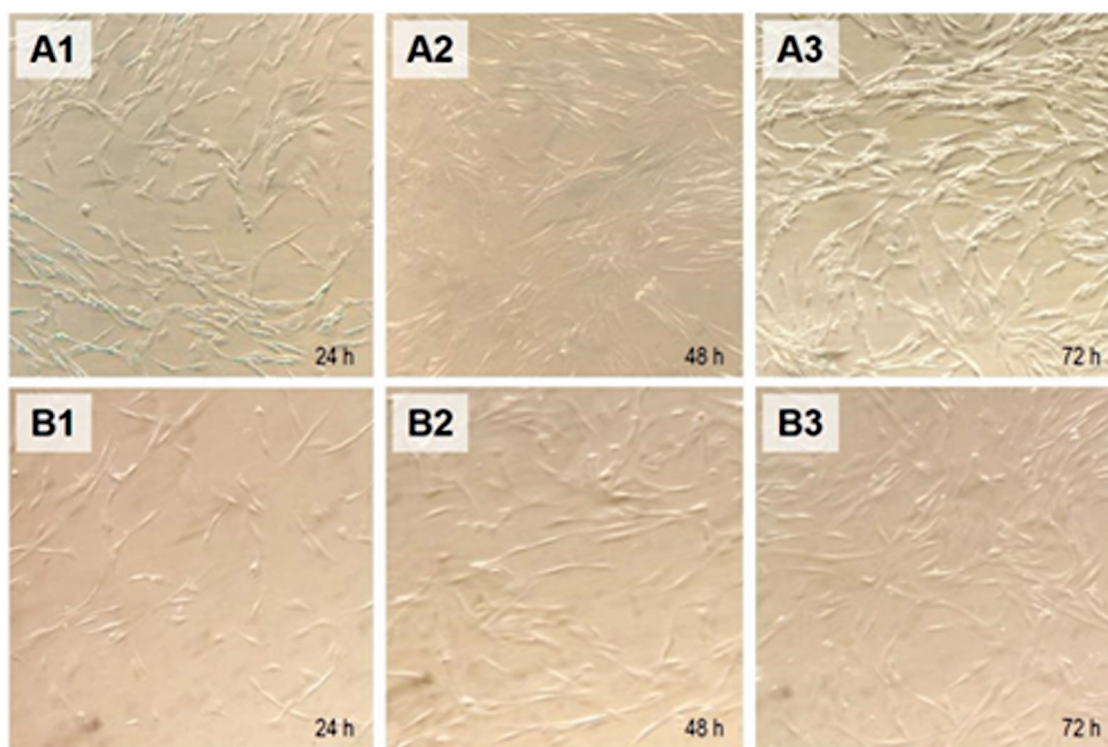
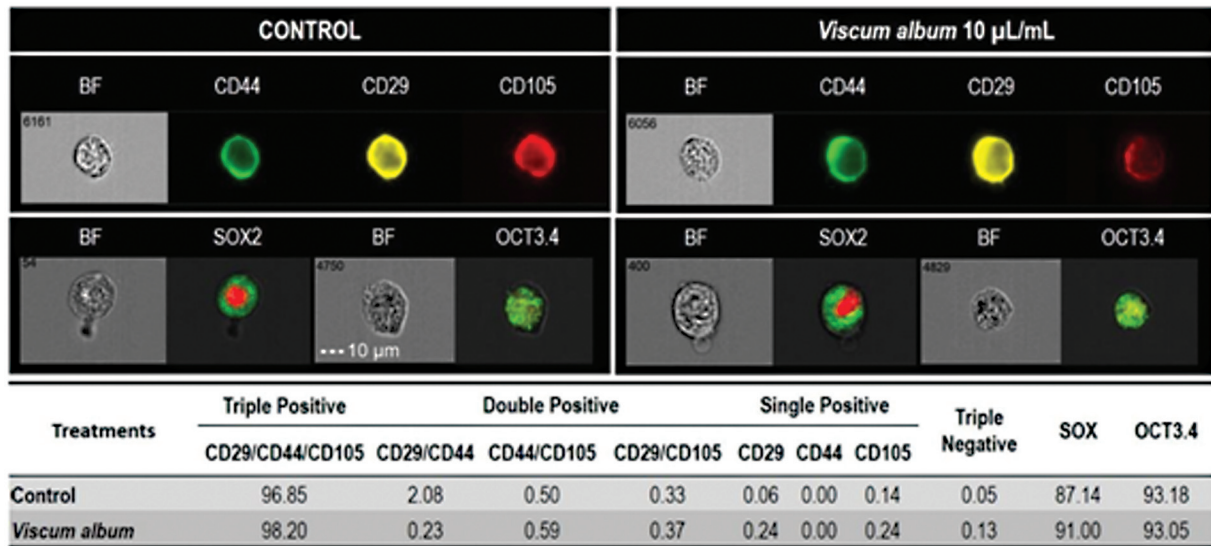**Fig. 3** Morphological characteristics of the MSC cultured *in vitro*, showing adhered cells with fibroblast-like format in the groups, control (**A**) and VA 10 µL/mL (**B**) after 24 hours (1), 48 hours (2), and 72 hours (3)

(b) Cell Differentiation in Multilineages

All the evaluated groups showed the expected morphology and positive markings for cell differentiation after induction in multilineages (chondrogenic, osteogenic and adipogenic), confirming their multipotency. Morphological changes that are typical of the chondrogenic, osteogenic and adipogenic differentiation process can be observed in ►**Fig. 5A, B**, and **C**, respectively, after cell exposure to VA at the concentration of 10 µL/mL. For chondrogenic differentiation (►**Fig. 4A**), MSC were positively labeled with Alcian Blue in the cell-matrix identification of chondrocyte glycosaminoglycans. After osteogenic differentiation, differentiated MSC presented positive staining for Alizarin Red (►**Fig. 5B**), demonstrating the formation of calcium deposits in the extracellular matrix. MSC exposed to the adipogenic differentiation medium were positively labeled with Oil Red (►**Fig. 5C**) on lipid droplets in adipocytes. The uninduced cells of the control group were maintained in culture for the same period of the multilineage differentiated cells and presented all negative marking characteristics with the three evaluated lineages.

Treatments	Triple Positive	Double Positive			Single Positive			Triple Negative	SOX	OCT3.4
	CD29/CD44/CD105	CD29/CD44	CD44/CD105	CD29/CD105	CD29	CD44	CD105			
Control	96.85	2.08	0.50	0.33	0.06	0.00	0.14	0.05	87.14	93.18
Viscum album	98.20	0.23	0.59	0.37	0.24	0.00	0.24	0.13	91.00	93.05

Fig. 4 Flow cytometry immunophenotyping of MSC after 72 hours of culture in the absence (control) and presence of VA at the concentration of 10 µL/mL (VA group) in *in-vitro* culture for 72 hours. Triple-, double-, and single-positive expression percentages of the CD44, CD29, and CD105 markers, and single-positive expression percentage of SOX2 and OCT3/4.

MTT Assay

As illustrated in ►**Fig. 6**, there was a significant and gradual decrease in cell viability as VA concentration increased, except for 30 and 40 µL/mL, which showed similar values. The presence of VA in the *in-vitro* culture medium had deleterious effects on cell viability, to a lesser extent when low concentrations were used.

Annexin-V Apoptosis Assay

To evaluate apoptosis in MSC after *in-vitro* culturing, an average of 4,000 cells per treatment were analyzed by flow cytometry, and the percentages of intact cells, and cells in initial and final apoptosis and necrosis, were quantified (►**Table 1**). A total of 92.0, 90.9, 96.2 and 91.5% of intact cells were obtained respectively in the control, diluent, VA (10 µL/mL) and VA (20 µL/mL) groups. Regarding the percentages of MSC in initial apoptosis, 8.1, 0.5, 1.6 and 2.3% were obtained respectively in the control, diluent, VA (10 µL/mL) and VA (20 µL/mL) groups. Additionally, the percentages of cells in final apoptosis were 0.1, 6.7, 1.7 and 5.4% respectively in the control, diluent, VA (10 µL/mL) and VA (20 µL/mL) groups. The number of cells in necrosis was below 2% in all treatments analyzed.

Discussion

The use of MSC in cytotoxicity tests constitutes an alternative model by which to predict initial doses of acute systemic toxicity, as recommended by international authorities, and so it is a promising *in-vitro* test.[30] In the present study, this
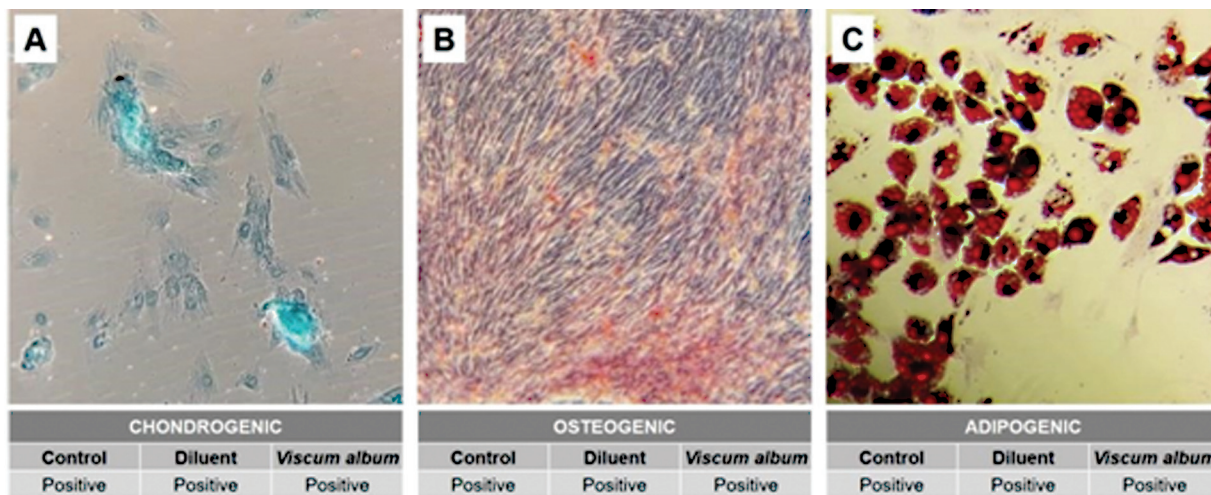
Fig. 5 *In-vitro* differentiation of MSC in multilineages after the 10 µL/mL VA treatment. (**A**) Blue coloration (*Alcian blue*) positive for alkaline phosphatase, showing cell differentiation for **chondrogenic** lineage. (**B**) Brownish marking (positive) demonstrated by Von Kossa staining in a mineral deposition, characterizing **osteogenic** differentiation. (**C**) Cytoplasmic lipid droplets observed in red color (*Oil Red*), demonstrating **adipogenic** differentiation.
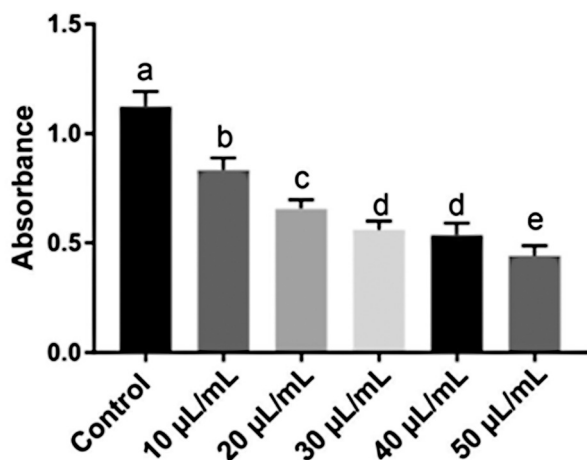
Fig. 6 MTT Assay in the absence (control) or presence of different VA concentrations (10, 20, 30, 40, and 50 µL/mL) in the MSC culture medium. Letters indicate significant differences between treatments ($p < 0.05$).

Table 1 Cellular apoptosis in control, diluent, and VA groups

Experimental group	Control	Diluent	VA 10 µL/mL	VA 20 µL/mL
Final apoptosis/ initial necrosis (%)	0.1	6.7	1.7	5.4
Initial apoptosis (%)	8.1	0.5	1.6	2.3
Necrosis (%)	0.0	1.7	0.8	0.6
Intact cells (%)	92.0	90.9	96.2	91.5

Abbreviation: VA, *Viscum album*.

model was used to evaluate VA D1D2 toxicity using concentrations between 10 and 50 µL/mL. This medicine seems to act directly on the multiplication potential of the cell by reducing the *in-vitro* growth kinetics of the canine MSC when used at a starting concentration of 20 µL/mL. Similarly, other studies reported the VA extract to be an efficient maturation inducer in human dendritic cells at concentrations evaluated as non-toxic (0.5 and 0.06 µg/mL).[31]

Homeopathic medicines may induce an active immune response, with low overexpression of inflammatory mediators compared to conventional medicines.[1,32] In the present study, the 10 µL/mL concentration maintained the cell viability and *in-vitro* proliferation potential, which may suggest the medicine's suitability for safety trials. This method of *in-vitro* study can be used as an initial screening in medicinal therapies, with a predictive capacity similar to other cell types (immortalized and neoplastic). It is a reasonable alternative due to its easy availability (of animal fat tissue), its physiological relevance (of self-renewal capacity), its *in-vitro* differentiation potential (multilineages), and the ease of cellular isolation and characterization.[30–32]

Regardless of its mechanism of action, the activity of VA in cancer cells, its clinical efficacy, and its survival benefit still need validation.[33] The use of VA originated as a treatment defined by anthroposophical medicine, which is indicated for cancer patients, whether or not in association with conventional treatments such as surgery, radiotherapy, chemotherapy or hormone therapy.[34] In the case of associated treatments, VA is used prior to conventional therapies with the aim of reducing adverse reactions. It can also be used later to improve the patient's quality of life.[35] *In-vitro* studies have shown that VA's action is based on the multimodal therapy concept, with a direct effect on tumor cells and modulation of supplemental pathways.[36] Improvement of the patient's health may be aided by immune system modulation through macrophage stimulation in the tumor microenvironment[37] and by suppression of carcinogenic effects due to anti-angiogenic activity.[38]

The use of other homeopathic medicines as complementary therapies has been reported but, as with VA, there have been few *in-vitro* studies. [39] *Thuja occidentalis*, *Carcinosinum* and *Ruta graveolens* have all been shown to have anti-neoplastic properties associated with immunomodulatory effects.[1] The latter effects include an increase in bone marrow cell proliferation and, more specifically, in the number of differentiated stem cells, showing stimulation of the hematopoietic system. Preparations of *T. occidentalis*, *Carcinosinum* and *R. graveolens* have been shown to possess anti-neoplastic activity in *in-vitro* tumor cell lineages in mice[2] by inducing apoptotic pathways mediated by the positive regulation of pro-apoptotic genes, such as p53 and caspases, with simultaneous downregulation of anti-apoptotic genes, such as Bcl-2.[40]

Mitochondrial activity, which was assessed in the MTT assay, was directly proportional to the increase in VA concentrations in the *in-vitro* tests using MSC. It was also confirmed by the Annexin-V-labeled apoptosis assays in the 10 µL/mL VA treatment. The induction of apoptosis is well defined through mechanisms exerted by lectins in VA.[40] Since lectins can inhibit protein biosynthesis at the ribosomal level, [41] the induction of initial apoptosis *in-vitro* could have been caused by the intracellular ribotoxic stress response pathways that directly affect mitochondria.

The inhibition of cell proliferation and apoptosis induction has been verified in a study analyzing the effects of VA extract at concentrations of 10 to 40 ng/mL in cancer cell lineages.[42] This potential cytotoxic effect of VA may be mediated by a cytokine-induced cellular apoptosis mechanism.[43] Though the present study did not evaluate *in-vitro* generated cytokines under VA stimulation in MSC, a significant decrease in cell viability was observed by MTT assays as the VA concentration increased. Apoptosis induction under VA action, as demonstrated in our study, may vary with the particular medicine preparation and cell type evaluated.[44–46]

When the VA concentration was increased to 20 µL/mL, MSC viability decreased. The number of cells in initial apoptosis increased, causing cell death, which may be due to the release of cytochrome C to the cytosol, along with proteolytic activation of caspase-8, -9, -3 and -2, with a consequent reduction in the expression of anti-apoptotic molecules, as described in other studies.[12,47,48] However,

VA's mechanisms of action require specific evaluation. Moreover, the origin of VA alters the composition of its active components, such as lectin, which is capable of enhancing VA cytotoxicity at high concentrations[49] by inhibiting the activity of the cytochrome P450 enzyme involved in oxidative biotransformation catalysis. However, that observation was only recorded in concentrations of VA extracts higher than 100 μg/mL.[50] Furthermore, the amount of lectins in the extract is capable of generating an immune response due to cytokine release.[51]

Despite the reduction of *in-vitro* growth kinetics at concentrations above 20 μL/mL and the reduction of MSC viability analyzed by the MTT assay in this study, the *in-vitro* development pattern of cells was preserved due to the expression of pluripotentiality and undifferentiation-related proteins in all treatments evaluated. In addition, the differentiation potential of MSC in multilineages was maintained when they were induced, which effectively characterizes the pattern of the cultured cell lineages. Due to their immunomodulatory effects, MSC constitute a group of cells with important activity for use in cellular therapies in degenerative diseases.[52] These cells are sensitive to various types of reagents during proliferation and differentiation, and are considered as an alternative for *in-vitro* testing of substances with tumorigenic activity.[53] In this study, *in-vitro* culture of MSC preserved the proliferation potential in the presence of a low concentration of VA (10 μL/mL), showing no direct cytotoxic effect.

This *in-vitro* study is the first to report that a low-diluted preparation of VA, often used in complementary cancer therapies, was capable of preserving the potential for cell multiplication without altering their characteristic undifferentiation. Irrespective of the *in-vitro* effect of VA on MSC viability, the potential for multilineage differentiation remained unchanged, even at concentrations above 10 μL/mL, which is considered to be toxic to cell viability and to induce apoptosis. This cell lineage is a promising alternative for *in-vitro* toxicity tests. It can be used for specific tissue tests when induced for differentiation, and it contributes to a precise evaluation of methods and compounds that promote cellular alterations according to the period of exposure and concentration.[54–57]

Cancer treatments include surgical removal, chemotherapy and radiotherapy. However, relapsing tumors are often resistant to conventional approaches.[42] This circumstance supports the judicious use of complementary therapies, which have been reported since 1920,[58] informed also by the promising effects of VA that have been observed since then on different tumor cell lineages in *in-vitro* and *in-vivo* studies, and also in clinical trials. However, systemic homeopathic treatment may interact with multicellular organisms and can interfere with the developmental pattern of both tumor and normal cell lineages. This potential interaction justifies the development of efficient alternative methods, using MSC that are capable of validating *in-vitro* studies for testing of modern therapies, as well as allowing the evaluation of species-specific lineage cells, whether in humans or animals,[59] and also here reported.

Other studies on the anti-tumor activity of VA, especially regarding its action in the tumor microenvironment and involving the release of immunomodulatory cytokines, need to be performed. Besides that, contributions involving the evaluation of molecular mechanisms, and controlled and randomized *in-vivo* studies, are also necessary.

Though the literature includes meta-analyses of randomized clinical trials which conclude that the clinical effects of homeopathy are superior to the placebo effect, there is still a lack of homeopathic clinical research data demonstrating the effectiveness of homeopathic treatment in a generalized way. The use of MSC as an *in-vitro* model for studying the effectiveness of homeopathic medicine helps resolve biases related to the placebo effect. Indeed, the major limitation of the present study is the lack of elucidation of the mechanism of action involved in the toxicity of VA D1D2 or of the characterization of the active compounds present in the VAD1D2 used. Therefore, in future studies involving toxicity and the induction of apoptosis by ultra-diluted VA, molecular analysis and characterization of the ultra-diluted VA preparations should be performed.

Conclusion

Though several studies have reported positive effects of homeopathic preparations of VA, *in-vitro* validations have not been available to date. Our results suggest that the use of efficient methods for evaluating *in-vitro* cytotoxicity of VA-based homeopathic medicine using MSC lineages may characterize this compound's potential action at different concentrations. Thus, *in-vitro* studies using different cell lineages may contribute to elucidating mechanisms that involve the effect of the medicine evaluated.

Highlights
- Mesenchymal stem cells are a reliable source of cells to evaluate cytotoxicity of drugs.
- The differentiation capacity of mesenchymal stem cells can be altered by cell treatments with low-diluted *Viscum album*.
- A low dilution of homeopathic *Viscum album* promoted a decrease in mesenchymal stem cell viability *in-vitro*.

Supplementary material

Supplementary file 1. Ethics Committee approval certificate

Authors' Contributions
A.C.V.V. conceptualized the study, and contributed to the methodology, investigation, formal analysis, and writing—original draft **H.d.S.S.B.** contributed to methodology, investigation, formal analysis, and writing—original draft. **B.S.L.D.** did the formal analysis and co-wrote the original draft. **L.S.R.** contributed to conceptualization, investigation, and acquisition of resources. **P.F.M.** contributed to

conceptualization and investigation, formal analysis, and writing—original draft. **R.R.** contributed to conceptualization and writing—review and editing. **R.A.C.** contributed to co-writing—review and editing. **R.V.A.** contributed to conceptualization, methodology, supervision, and project administration. All authors critically reviewed the manuscript, contributed to its revision, and approved the final version submitted.

Conflict of Interest
None declared.

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