Proliferation of fibroblasts and endothelial cells is enhanced by treatment with *Phyllocaulis boraceiensis* mucus

A.R. Toledo-Piza*, E. Nakano†, R.E.G. Rici‡ and D.A. Maria*

*Laboratory of Biochemistry and Biophysics, Butantan Institute, Av. Vital Brazil, 1500, 05503-900, São Paulo, Brazil, †Laboratory of Parasitology, Butantan Institute, Av. Vital Brazil, 1500, 05503-900, São Paulo, Brazil, and ‡School of Veterinary Medicine, University of São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, 05508-270, São Paulo/SP, Brazil

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Abstract

Previously, mucus of some molluscs has been studied as a potential source of new natural compounds capable of inducing cell proliferation and of remodelling tissue. Here, the focus of the study is possible use of mucus released by *Phyllocaulis boraceiensis* – a compound inducing cell proliferation and enhancing collagen synthesis in dermal fibroblasts and inducing proliferation human endothelial cell cultures. Fibroblasts treated with *P. boraceiensis* mucus at concentrations below 0.012 µg/µl developed high rates of proliferation, as evaluated using MTT assay; the proliferative effect was dose-dependent. Production and secretion of extracellular matrix components and collagen type I fibres were enhanced after 24 h of treatment, revealing a hormesis effect, biphasic dose response – low dose for proliferation yet toxic at high dose. No significant change in proliferation was observed in treated endothelial cells and production of lipid polyunsaturated free radicals was low in both cell types. Treatment with *P. boraceiensis* mucus produced pronounced changes in fibroblast cell number and morphology, and in quantities of well-ordered collagen deposition. These results support the premise that *Phyllocaulis boraceiensis* mucus demonstrates proliferative properties in cells involved in the healing process.

Introduction

As a consequence of recent attempts to prospect for natural sources for biotechnological use and drug discovery, it is becoming clear that many natural compounds (ligands) are able to reach receptor sites on or within cells (1). Many of such natural products are found in invertebrates such as molluscs (2), the most successful example of which is of *Conus magus*, a marine mollusc that releases a peptide efficient in controlling terminal phase pain in HIV-infected individuals and in cancer patients (3). This peptide, ziconotide, was approved in the United States in 2004 under the trade name Prialt® as an analgesic drug for treatment of chronic pain in spinal cord injury (4,5). Terrestrial invertebrates have a long history as a source of bioactive compounds, as is the therapeutic effect of *Helix aspersa* mucus also (6). This study listed several instances of use of snails to treat burns, abscesses, stomach pain and of nephritis, dating to 1700 and earlier, mentioning the philosophers Celse and Pliny (the Elder) – although their theories lacked scientific proof of efficacy. Thus, mankind has long considered molluscs to be a surprisingly robust source of compounds with biological and therapeutic properties.

In a study using pure mucus of *Achatina fulica* applied on injured rabbit skin, clinical and histological analysis demonstrated faster healing processes and abundant presence of collagen fibre (7). In breeding of the snail *Helix aspersa* Müller in Chile, the animal handlers claimed that mucus released by these animals acted to heal wounds on their hands (8). To verify this healing property, experimentation was performed to compare cicatricial activity of two creams made of mucus of this snail, Elicina® and Novobase®, the study involving 36 patients divided into two groups of 18, who had burn scars and grafts on their face, neck and hands. Authors noted that 14 patients treated with Elicina® had higher

Correspondence: D.A. Maria, Laboratory of Biochemistry and Biophysics, Butantan Institute, Av. Vital Brazil, 1500, Zip code: 05503, 900, São Paulo, Brazil. Tel.: 55-11-26279750; E-mail: durvanei@usp.br

partial pigmentation of the grafts, seven showed reduction in size of scars, and three had changes in texture of the graft. In the group treated with Novobase[®], one patient had partial depigmentation of the graft, with reduction in scarring, but no patient demonstrated changes in texture of the graft. This indicates that some creams that contain mucus of snails are effective in some treatments of burns. In a study with 27 patients suffering from partially deep burns on the face it was observed that the mean period of endothelial reconstruction was 11 ± 2 days for those treated with Elicina[®] and 15 ± 2 days for the control group. *Helix aspersa* mucus speeds up apoptotic process in burned tissue and epithelialization of deep partial burns (9).

Wound healing is a dynamic set of tissue changes important for maintaining integrity of an organism; it involves inflammation, chemotaxis, cell proliferation and differentiation and tissue remodelling (10,11). Wound healing is a necessary component of the repair process, providing mechanisms by which injured tissue is prepared for reconstruction. This phenomenon is usually multimediated, dynamic, transient and self-limited. However, when an inflammatory stimulus cannot be eliminated or removed, a complex response involving the whole body and leading to chronic inflammation (that can often be deleterious) is triggered (12).

The mucus of *Phyllocaulis boraceiensis*, a Brazilian slug, is being studied as a source of new therapeutic compounds (13); mucus of this species is composed of a complex mixture of components (14). The mucus is released by skin gland cells located in the rear pedal region (15) and functions to prevent desiccation (16), reduce friction in locomotion (17) and protect the slug's body against mechanical injuries and noxious substances (18). Modern societies have become more cognizant of holistic and natural treatments of disease and are now embracing development of such products (19). Although some people view these ideas to be somewhat primitive or ignorant, many such remedies are the result of thousands of years of empiric observation – which emphasizes the importance of scientific studies to confirm action of these natural products in wound healing.

To understand how normal human cells behave when treated with *Phyllocaulis boraceiensis* mucus, we studied cell synthesis and proliferation capability of mucus-treated fibroblast and endothelial cell cultures.

Materials and methods

Collection and preparation of mucus

The specimens of *Phyllocaulis boraceiensis* (20) originated from captive breeding at the Butantan Institute

(São Paulo, Brazil). Animals were kept in plastic boxes containing soil *in natura* in a pollution-free and temperature-controlled laboratory environment at 24 °C and 85% relative humidity. Daily, they were fed small amounts of a variety of vegetables (21) and before collection of mucus, animals were transferred to a Petri dish and nebulized with distilled water, to remove particles of soil and plants from their bodies, using forceps when necessary (22).

Mucus was obtained by stimulating pedal tissues. Animals were maintained freely moving on the glass plate with saline solution (NaCl-0.06%) for 5 min (13). Secretions were collected using a spatula and stored in plastic tubes at -80 °C. Experiments were performed using lyophilized samples hydrated in organic solvent: 1 mg of lyophilized mucus, 100 ml of monoamine, 350 μ l of ethanol and 500 μ l of methanol, supplemented with distilled water to 6 ml.

Protein determination

Protein concentration was determined using Bradford's method (23) with bovine serum albumin as standard (Bio-Rad Co - USA). Samples of lyophilized mucus solubilized in organic solvents were analysed, and absorbance was measured in a spectrophotometer (Pharmacia Ultrospec 2000) at λ 595 and 280 nm; and concentrations were determined with the aid of a standard curve.

Cell culture

Dermal fibroblast normal (ATCC-PCS-201-010) and endothelial cell (ATCC-CRL-1730) cells were cultured in 75 cm² culture bottles in RPMI-1640 medium supplemented with 10% inactivated foetal bovine serum, 2 mM of L-glutamine and antibiotics, and incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were observed three times a week, and at these same times, medium was changed totally. Detached cells were centrifuged twice and rehydrated in growing medium; cell concentration was adjusted to 5×10^6 cells/ml and counted in a Mallassez chamber. Morphological aspects of fibroblast and endothelial cells treated with *P. boraceiensis* mucus were observed using an inverted light microscope (Olympus CKX41); a digital camera (Sony DSC 300) was used to record digital micrographs.

Determination of cell cycle phases

Flow cytometry was applied to determine kinetic parameters of the cell populations and to provide percentages of cells that were in G0/G1, S, and G2/M phases, as well as those undergoing apoptosis or DNA fragmenta-

tion (24). These human normal fibroblasts and endothelial cells, treated with 100 µl of 0.18, 0.09, 0.045 and 0.0007 µg/µl *P. boraceiensis* mucus for 24, 48 and 72 h, were transferred to flow cytometry tubes and stained with propidium iodide. Cells were counted at different cell cycle phases using a FACSCalibur (BD, MA, USA) flow cytometer.

Determination of proliferative response

The normal dermal fibroblasts and endothelial cells, treated with 100 µl of *P. boraceiensis* mucus (total protein at different concentrations 0.18–0.0007 µg/µl), were seeded at a density of 10^5 cells/well in 96-well tissue culture plates (Corning Inc., NY, USA) for 24, 48 and 72 h. Measurements of cell proliferation were obtained using the MTT method (25). MTT is a widely accepted method that utilizes reduction of tetrazolium salts in evaluation of both cell proliferation and cell death. Yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, partly by action of dehydrogenase enzymes, to generate reducing equivalents of NADH and NADHP. Resulting intracellular formazan was solubilized and quantified by spectroscopic means. After fibroblast and endothelial culture medium elimination and dissolution of the crystals by dimethyl sulphoxide, a spectrophotometer (Biorad, CA, USA) was used to measure optical density at 570 nm, this is directly proportional to number of live cells.

Determination of cell division rates following mucus treatment in fibroblasts and in endothelial cells

Proliferation determination by CFSE (5,6-carboxyfluorescein diacetate succinimidyl ester) labelling method was adapted from a previously described protocol (26)

Figure 1. Dose-response relationship of normal mouse lymphocyte proliferation (used as a control group) measured using the CFSE labelling method.

that allows for direct detection of single proliferating cells, and facilitates quantification of cell division by flow cytometry, according to respective CFSE-dilution.

Samples of human normal fibroblasts and endothelial cells treated with 0.18, 0.09 and 0.045 µg/µl of *P. boraceiensis* mucus total protein for 24, 48 and 72 h were analysed. Lymphocytes of Balb-c mice were used as a control group of phytohaemagglutinin-mitogen-stimulated cells. The principle of analysis of lymphocyte proliferation is as follows: CFSE is divided equally into daughter cells following cell division, and intensity of fluorescence is half that of the parental generation. Thus, in a whole cell population that is undergoing proliferation, fluorescence intensity declines by half in the following generation. Methods used to perform proliferation analysis using CFSE have been described previously (27–30). CFSE flow cytometric data files were analysed using CellQuest™ acquisition/analysis software (Becton Dickinson, San Jose, CA). Fifty thousand events were collected, and population proliferation was analysed using ModFitLT 2.0 software (Proliferation Wizard Methods).

Lymphocyte proliferation assay: Lymph nodes of two normal Balb-c mice were cultured in RPMI-1640

Figure 2. Dot plot representing a flow cytometry analysis of CFSE-labelled mouse lymphocytes from lymph nodes normal as follows: (a) without treatment with 10 µg/ml phytohaemagglutinin and (b) with treatment with 10 µg/ml phytohaemagglutinin (mitogenic stimulation)

medium supplemented with 2 mM L-glutamine (Cambrex, Belgium), 500 μ M 2-mercaptoethanol (Sigma Aldrich, Germany), 10% heat-inactivated FCS and 1% penicillin/streptomycin (Cultilab, Brazil) at final concentration of 10^6 cells/ml. Cell (10^5) suspensions were cultured in U-bottom 96-well plates (Nunc, Denmark) with either 10 μ g/mL phytohaemagglutinin (PHA) (Sigma Aldrich, Germany) or medium alone. In each case a pellet of 10^6 cells was resuspended in 1 ml of CFSE labelling solution with concentrations ranging from 10 μ M to 37 nM and incubated for 10 min at 37 °C in the dark. After labelling, lymphocytes were incubated at 37 °C in a 5% CO₂ incubator for 48–96 h. Samples were transferred to flow cytometry tubes, and cells were counted using a FACSCalibur (BD – USA) flow cytometer (Figs 1, 2a and b).

Determination of collagen production

Collagen production of human normal fibroblasts and endothelial cells treated with 100 μ l of 0.18 μ g/ μ l *P. boraceiensis* mucus in serial dilution for 24, 48 and 72 h, was measured using the Picrosirius method (31), reported to be a specific method for collagen detection (32). This utilizes staining collagen and differentiation between distinct forms of collagen fibres (33). Samples were stained for 1 h in 0.1% Sirius Red (Sigma-Aldrich Chemical). Dye was dissolved in saturated aqueous picric acid at 100 mg/100 ml. Bouin's fluid for cell fixation was prepared by mixing 15 ml of saturated aqueous picric acid with 5 ml of 35% formaldehyde and 1 ml of glacial acetic acid. Both reagents are stable for months, but we used freshly prepared dye reagent for each experiment. Cells were stained for 1 h with mild shaking on a microplate shaker. Thereafter, the dye solution was removed by suction, and stained cell layers were washed extensively with 0.01 N hydrochloric acid to remove all non-bound dye. Cell morphology was photodocumented before dissolving the stain. When 24-well

Figure 3. Comparison of microscopic aspects of cultures submitted to different concentrations of mucus of *Phyllocaulis boraceiensis*. (a) Microscopic analysis of human dermal fibroblast primary cultures. Note the elongated cells that are spindle-shaped, divided into bundles, rearranged and adhered to the culture plate. (b) Microscopic analysis of human dermal fibroblast primary cultures treated with 0.045 μ g/ μ l of mucus of *Phyllocaulis boraceiensis*. Note the elongated cells that are spindle-shaped, divided into bundles, rearranged with high cell density (filled arrow) and adhered to the culture plate. (c) Microscopic analysis of human endothelial cell primary cultures. Note the elliptical cells that are distributed in bundles, rearranged and adhered to the culture plate. (d) Microscopic analysis of human endothelial cells primary cultured with 0.18 μ g/ μ l of mucus of *P. boraceiensis*. Note the evidence of cellular death. Photomicrographs using an inverted microscope

Figure 4. Effects of *Phyllocaulis boraceiensis* mucus on fibroblast (a–c) and endothelial cell (d–f) cycle phase as assayed by flow cytometry. Cells were left untreated or were treated with 0.18, 0.09, 0.045 or 0.0007 µg/µl of *P. boraceiensis* mucus for 24, 48 and 72 h. The results are expressed as the mean percentage of cells at different phases of the cell cycle: G0/G1, S and G2/M. Data are presented as the means $\pm$ SD of triplicate experiments. ns = non significant, * P < 0.05, ** P < 0.005, and *** P < 0.0001 compared to the control

culture dishes were used, stained material was dissolved in 0.2–0.3 ml of 0.1 N sodium hydroxide using a microplate shaker for 30 min at room temperature. Dye solution was transferred to microtitre plates, and optical density was measured using a spectrophotometer (BenchMark, Biorad) with wavelength adjusted at 550 nm against 0.1 N sodium hydroxide as a blank.

Determination of lipid peroxidation

Lipid peroxidation of the human normal fibroblasts and endothelial cells, treated with different concentrations of *P. boraceiensis* mucus total protein for 24, 48 and 72 h, was assayed by utilizing malondialdehyde as a thiobarbituric acid (TBA)-reactive substance (34). In brief,

Figure 5. Viability/cellular proliferation evaluation using the MTT test in human normal fibroblasts (a) and endothelial cells (b) treated with 0.18 µg/µl of *Phyllocaulis boraceiensis* mucus in serial dilution

H₃PO₄ (1%) and aqueous TBA solution (0.6%) were added to the 10% homogenate. The mixture was shaken and heated using a boiling-water bath for 45 min. After cooling, *n*-butanol was added, and the mixture was shaken. After separation of the butanol layer by centrifugation at 1200 *g* for 15 min, its optical density was determined using a spectrophotometer (Biorad – USA), with wavelength adjusted to 530 nm. The difference between results of the two optical density determinations was taken as the TBA value, and amounts of malondialdehyde (MDA) produced were calculated and compared to MDA standards and expressed as µMol MDA.

Scanning electron microscopy

The human normal fibroblasts and endothelial cells treated with 0.18, 0.045 and 0.0007 µg/µl of *P. boraceiensis* mucus total protein for 24 h, were rinsed twice in PBS and fixed in 3% glutaraldehyde in PBS, overnight

at room temperature. After being washed three times in PBS, samples were fixed in 1% osmium tetroxide (pH 7.4) at 4 °C for 1 h. They were then dehydrated, dried in a critical point dryer and gold sputtered. Scanning electron microscope (SEM) images were taken on a LEO 435 VP SEM (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad, CA, USA), and statistical significance of differences between groups was determined using unpaired one-way ANOVA. Means were compared using Tukey's multiple comparison tests. Regression analysis was used to examine data obtained and to infer relationships between a dependent variable and an independent variable. Comparisons were considered either non significant (ns) or significant at **P* < 0.05, ***P* < 0.005 or ****P* < 0.0001.

Results

Experiments were conducted using fixed concentration of 0.18 µg/µl of *Phyllocaulis boraceiensis* mucus, determined using the Bradford method, in serial dilutions (0.18, 0.09, 0.045, 0.023, 0.012, 0.006, 0.003, 0.0015 and 0.0007 µg/µl) applied to human normal fibroblast and endothelial cell cultures that were collected 24, 48 and 72 h after treatment. Results of the MTT colorimetric assay confirmed that *Phyllocaulis boraceiensis* mucus was caused a significant proliferative response in normal human fibroblasts and endothelial cells treated with low concentrations of mucus, and that cell population growth was also stimulated, compared to controls, after 72 h.

Using an inverted microscope (40× magnification), cytological analysis was performed on the human dermal fibroblast primary cultures (Fig. 3a) and cultures treated with 0.045 µg/µl of *P. boraceiensis* mucus total protein (Fig. 3b). Mucus-treated cells appeared elongated, spindle-shaped and divided into bundles, rearranged with high cell density and adhered to culture plates. Endothelial cells were also analysed; control endothelial cells (Fig. 3c) were compared to endothelial cells treated with 0.18 µg/µl of *P. boraceiensis* mucus total protein after 48 h of treatment (Fig. 3d). We observed cell death in these mucus-treated cultures.

The study of cell cycle phases (shown here in graphics) illustrates comparison of percentages of human normal fibroblasts (Fig. 4a–c) and endothelial cells (Fig. 4d–f) that were in G0/G1, S, G2/M phases, and exhibited DNA fragmentation. This experiment was conducted using original concentration in serial dilutions, although

Figure 6. Histograms represent the flow cytometric analyses of CFSE-labelled fibroblasts (a) and endothelial cells (b) treated with 0.18, 0.09 and 0.045 $\mu\text{g}/\mu\text{l}$ of *Phyllocaulis boraceiensis* mucus for 24, 48 and 72 h

we show only the extreme results here; thus, only significant concentrations (0.18, 0.09, 0.045 and 0.0007 $\mu\text{g}/\mu\text{l}$) of *P. boraceiensis* are presented, to ease viewing of results.

MTT colorimetric assay revealed high levels of proliferative response of the human normal fibroblasts (Fig. 5a), primarily at concentrations less than 0.012 $\mu\text{g}/\mu\text{l}$, and were revealed to be dose-dependent. Concentrations greater than 0.012 $\mu\text{g}/\mu\text{l}$ exhibited toxicity. Endothelial cells (Fig. 5b) did not display any significant change in proliferation after 24 h of treatment compared to controls. After 48 h treatment, proliferation rates were 50% higher than those presented by controls, displaying a dose-dependent response. Cells that were subjected to 72 h of *P. boraceiensis* mucus treatment revealed its toxicity.

Lymphocytes were labelled with CFSE to be used as control groups and were cultured for 48, 72 and 96 h; after culture, cells were harvested and analysed by flow cytometry. Cell division is characterized by sequential halving of CFSE fluorescence, generating equally spaced peaks on a logarithmic scale; peaks indicate the division cycle number. Similar results of MTT colorimetric assay were obtained using CFSE methodology, which accurately confirmed rate of proliferation of normal human fibroblasts and cytotoxic effects to endothelial cells, in different periods of treatment with *P. boraceiensis*

mucus. Figure 6 presents histograms acquired using ModFitLT 2.0 software, and comparison of significant differences is shown in Fig. 7.

Fibroblasts are mesenchymal cells that play a critical role in embryonic morphogenesis, wound repair, and tissue remodelling, by synthesizing and organizing connective tissue components. Here, human normal fibroblasts developed increased collagen production by over 6-fold (Fig. 8a), mainly at mucus concentrations between 0.09 and 0.023 $\mu\text{g}/\mu\text{l}$, while endothelial cells did not display any significant production of collagen (Fig. 8a), retaining basal levels. Lipid peroxidation, or free radical production, as assayed by measuring malondialdehyde production, presented a significant reduction when compared to levels produced by controls. Results are shown in Fig. 9a for human normal fibroblasts and in Fig. 9b for endothelial cells.

Surface morphologies of fibroblasts (Fig. 10 a–d) and endothelial cells (Fig. 11 a–d) treated with 0.18, 0.012 and 0.0007 $\mu\text{g}/\mu\text{l}$ of *P. boraceiensis* mucus for 24 h, were investigated by scanning electron microscopy.

Discussion

The experiments described here were designed to provide knowledge concerning mechanisms behind effect of

Figure 7. Proliferation rates in fibroblasts (a) and endothelial cells (b) treated with *Phyllocaulis boraceiensis* mucus for 24, 48 and 72 h determined using the ModFitLT 2.0 software

mucus of *Phyllocaulis boraceiensis* on proliferation of fibroblasts and endothelial cells. Thus, comparisons to results of non-scientific studies of mucus from molluscs such as *Helix aspersa* and *Achatina fulica* (both exotic species in Brazil), known to be producers of mucus capable of renovating burned tissues, could be made (8,9).

The activity of *Phyllocaulis boraceiensis* mucus, on proliferation of human normal cells, was studied using cell cycle phase analysis to compare results obtained between cells treated with mucus and those not treated. Transition from G1 to S phase requires assembly and activation of a DNA replication complex to initiate DNA synthesis. Endothelial cells undergo contact inhibition, prohibiting their proliferation at confluence, and become quiescent. The cell cycle in eukaryotes controls progression, between and within the phases, through checkpoints that coordinate proliferation of the cells with the surrounding environment (35), and help ensure accuracy of DNA replication and division (36).

Figure 8. Collagen produced by human normal fibroblasts (a) and endothelial cells (b) treated with 0.18 µg/µl of *Phyllocaulis boraceiensis* mucus in serial dilution

Arrest of cell cycle stages was seen to be dependent on mucus concentration, and increase in S phase was observed in human fibroblasts treated for 24–48 h, maintained up to 72 h treatment. In endothelial cells, treatment with mucus induced G2/M arrest; this phenomenon can occur by a variety of mechanisms.

Measurement of cell proliferation with CFSE offers many advantages over conventional ^3H -thymidine incorporation assays. Labelling cells with CFSE is a simple procedure that eliminates use of radioactive materials and results in an extremely bright fluorescent signal that is easily detected by table top flow cytometers. Intracellular esterase hydrolyses CFSE into a fluorescent dye that binds covalently to cytoplasmic amino acid residues such as lysine (37); thus, only viable cells are labelled. However, labelled cells that die during the culture period remain detectable until they disintegrate. These late apoptotic cells and cell fragments can cause overall

Figure 9. Free radicals produced by human normal fibroblasts (a) and endothelial cells (b) treated with 0.18 $\mu\text{g}/\mu\text{l}$ of *Phyllocaulis boraceiensis* mucus in serial dilution

increased and less well-defined CFSE signals that may artificially affect results. Values obtained with the proliferative index determined by CSFE assay showed that effects on proliferative response of fibroblasts and endothelial cells when treated with *Phyllocaulis boraceiensis* were similar to those results obtained by MTT colorimetric assay.

The extracellular matrix (ECM) plays an important role in determining cell and organ function; it is an organizing substrate that provides tissue tensile strength, it anchors cells and influences cell morphology and function (*via* interaction with cell surface receptors), and it is a reservoir for growth factors (38). Overproduction of collagen by normal dermal fibroblasts demonstrates the capability of *P. boraceiensis* mucus to be a satisfactory inducer of tissue regeneration. This result can be compared to a new technique of suture-less prepuceplas-

Figure 10. Morphological changes of fibroblast cells observed by scanning electron microscopy (SEM). (a) General view of the cell density in the control group. (b) After fibroblast exposure to 0.18 $\mu\text{g}/\mu\text{l}$ of *P. boraceiensis* mucus for 24 h, the treatment was found to be toxic; (c) preserved extracellular matrix after exposure to 0.012 $\mu\text{g}/\mu\text{l}$ of *Phyllocaulis boraceiensis* mucus; (d) cells treated with 0.0007 $\mu\text{g}/\mu\text{l}$ showed high cell confluence.

Figure 11. Morphological changes of endothelial cells observed by scanning electron microscopy (SEM). (a) General view of the cell density in the control group; (b) cells treated with 0.18 µg/µl showed toxicity; (c) cells treated with 0.012 µg/µl showed enhanced proliferation capability and organisation of extracellular matrix fibres; (d) preserved extracellular matrix and blood vessel formation after exposure to 0.0007 µg/µl of *Phyllocaulis boraceiensis* mucus

ty using 'Elicina[®]', a cream composed of mucus of *Helix aspersa* that contains allantoin, collagen, elastin, glycolic acid and vitamins A, D, and E and has presented an acceleration in the healing process (39). Additionally, as mentioned here before, creams composed of mucus of *H. aspersa* accelerated the process of apoptosis in burned tissue and improved epithelialization of deep partial burns (9). Unfortunately, no scientific citations have provided precise collagen quantification, due to which it is impossible to establish a comparison between *P. boraceiensis* and *H. aspersa* mucus.

Fibroblasts are the foremost cells involved in wound healing and whose main function is to maintain the integrity of tissue, by synthesis of extracellular matrix components. They can be stimulated to do this and deposit extracellular matrix components; concerning skin, these events are necessary to enable and promote re-epithelialization (40). *P. boraceiensis* mucus induces fibroblast proliferation and causes them to synthesis more collagen to remodel extracellular matrix, over a limited time. This procedure is usually not feasible over long periods as proliferation of fibroblasts is closely linked to accelerated cell death and DNA damage, caused by free radicals.

Both high rates of cell proliferation and extensive production of collagen (700 µg/µl) in fibroblast cultures treated with *P. boraceiensis* mucus suggest that this compound can act as a wound-healing agent. In sum, increase in fibroblasts increases collagen production and extracellular matrix remodelling. Thus, drugs with capabilities to induce this are very interesting when the focus is on speeding up tissue regeneration and improving quality of life and recovery of burn patients or those presenting with difficult-to-close wounds. Endothelial cells and fibroblasts treated with *P. boraceiensis* mucus present a significant reduction in production of free radicals when measured using malonaldehyde rates. Unsaturated lipid molecules of cell membranes are particularly susceptible to damage by free radicals and readily contribute to uncontrolled chain reactions.

Studies in animals have shown that ageing is frequently associated with accumulation of oxidized forms of proteins (41). Excessive production of free radicals causes damage to biological material and is an essential event in aetiopathogenesis of various diseases. It can lead to defective energy production or possibly increases free radicals in cells, resulting in apoptosis (42). Results obtained show that wound-healing treatments involving proteins of mucus of *Phyllocaulis boraceiensis* are recommended to show significant reduction in production of free radicals.

Rates of proliferation of human normal fibroblasts show that *Phyllocaulis boraceiensis* mucus acts as an

inducer of cell growth at low concentrations, suggesting that large quantities of protein from *P. boraceiensis* mucus is not necessary. High proliferation in endothelial cells was observed only after 48 h of treatment. The differential response of fibroblast and endothelial cells implied a hormesis effect, defined as a term used in toxicology studies to describe a biphasic dose response with low-dose stimulation (beneficial effect) and high-dose inhibition (toxic effect) (43). The response of a cell or organism to the low dose of the compound is considered an adaptive compensatory process following an initial disruption in homeostasis.

To conclude, these results support the premise that *Phyllocaulis boraceiensis* mucus has proliferative properties when applied, and enhances collagen synthesis, in cells involved in healing, making this compound an effective model for development of molecules with low production costs yet great specificity.

Conflicts of interest

The author(s) declare that there are no conflicts of interest.

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