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Atropa Belladonna L. Water Extract: Modulator of Extracellular Matrix Formation in Vitro and in Vivo

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Summary

Previously, we found that treatment of cutaneous wounds with *Atropa belladonna* L. (AB) revealed shortened process of acute inflammation as well as increased tensile strength and collagen deposition in healing skin wounds (Gál *et al.* 2009). To better understand AB effect on skin wound healing male Sprague-Dawley rats were submitted to one round full thickness skin wound on the back. In two experimental groups two different concentrations of AB extract were daily applied whereas the control group remained untreated. For histological evaluation samples were removed on day 21 after surgery and stained for wide spectrum cytokeratin, collagen III, fibronectin, galectin-1, and vimentin. In addition, in the *in vitro* study different concentration of AB extract were used to evaluate differences in HaCaT keratinocytes proliferation and differentiation by detection of Ki67 and keratin-19 expressions. Furthermore, to assess ECM formation of human dermal fibroblasts on the *in vitro* level fibronectin and galectin-1 were visualized. Our study showed that AB induces fibronectin and galectin-1 rich ECM formation *in vitro* and *in vivo*. In addition, the proliferation of keratinocytes was also increased. In conclusion, AB is an effective modulator of skin wound healing. Nevertheless, further research is needed to find optimal therapeutic concentration and exact underlying mechanism of action.

Key words

Phytotherapy • Aqueous extract • Wound healing • Fibroblast • Keratinocyte • Inflammation

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Introduction

The quest for finding ways to improve wound healing warrants to test new approaches. Toward this end, experimental studies are exploring the potential for benefit, examining for example physical (Gurdol *et al.* 2010, Lacjakova *et al.* 2010, Toporcer *et al.* 2006, Vasilenko *et al.* 2010) and pharmacological methods (Chu *et al.* 2010, Gál *et al.* 2010, Novotný *et al.* 2011), stem cell therapy (Luo *et al.* 2010, Nishino *et al.* 2011) or phytotherapy (Priya *et al.* 2004, Skórkowska-Telichowska *et al.* 2010). Considering economic aspects, applying natural products represents a feasible option of treatment in many regions of the world. In this respect, the use of *Atropa belladonna* L. (AB) aqueous extract for improving skin-wound healing has a long

tradition in the folk medicine of "Spiš region" located in the Eastern Slovakia. As often encountered, the effect of this herb has never rigorously been experimentally verified, prompting us to perform our previous investigation. We found that AB-treated wounds have a shortened process of inflammationas as well as enhanced collagen deposition and increased tensile strength when compared with their untreated controls (Gál *et al.* 2009). Furthermore, our *in vitro* study revealed that keratinocytes in the presence of AB expressed keratin-19.

Poorly differentiated cells have a large potential to stimulate new tissue development and/or formation (Chen *et al.* 2009, Fu and Li 2009), an aspect of crucial significance for wound healing (Lau *et al.* 2010). Herein, several (glyco)proteins, especially components of the extracellular matrix (ECM), play important roles to facilitate cell-cell and cell-matrix interactions, what is essential for an effective course of healing (Suzuki *et al.* 2003, Nečas *et al.* 2010, Dvořánková *et al.* 2011). It has already been shown that an ECM rich in fibronectin and galectin-1 may serve as active substratum that can substitute for feeder cells in the case of keratinocytes (Dvořánková *et al.* 2011). In addition, galectin-1, an endogenous adhesion/growth-regulatory lectin (Gabius 2006, Villalobo *et al*. 2006, Gabius *et al*. 2011) was found to be up-regulated during the early phases of healing (Klíma *et al.* 2009) as well as to be capable to act anti-inflammatorily (Cooper *et al.* 2010). Of note, both fibronectin and galectin-1 production have been detected in our *in vivo* and *in vitro* investigations. To extend the scope of study, collagen-3, keratin-19, Ki67, vimentin, and wide-spectrum keratin were investigated as well.

In principle, in order to achieve swift repair and regeneration of injured tissues it would be ideal to prime cells for optimal cell biological properties and for suitable ECM production. In our previous investigation, it was observed that AB has the capability to modulate these processes (Gál *et al.* 2009). This study was therefore designed as a combined *in vivo* (conducted on rats) and *in vitro* (conducted on keratinocytes and fibroblasts) investigation to better understand the mechanisms behind AB-dependent modulatory effects on skin wound healing. Tissue and cell specimens were processed under identical conditions to exclude any factor other than AB treatment to affect signal occurrence and intensity.

Methods

Plant material – Atropa belladonna L. (AB)

AB (*Solanaceae*) was collected in August 2006 from the vicinity of mast on Čertova sihoť, Slovak Paradise, Slovak Republic. The plant was unequivocally identified by Dr. Pavol Mártonfi from the Department of Botany, Institute of Biology and Ecology, Šafárik University in Košice. Herb (overground parts) of the plant was dried at room temperature in the dark. A voucher specimen (KO-30301) was deposited in the Herbarium of the Botanical Garden of the Šafárik University in Košice.

Preparation of the aqueous extract of AB

The water extract for both *in vitro* and *in vivo* experiments was prepared by pouring 10 g of a powder of dried AB leaves into 100 ml of boiling distilled water. The suspension was then left for 10 minutes at room temperature. Consecutively, the extract was filtered (0.2 μm).

For the animal study two concentrations were used: the original solution obtained by extraction (AB-10 %) and a 10-times diluted concentration (AB-1 %). For the *in vitro* study the extract was 10-times diluted in culture medium and the obtained concentration was considered to be the highest concentration (AB-1 %). In addition to this concentration tested, AB extract was 256 times diluted (AB-0.00390625 %). Preliminary dose-response testing included 0.25 %, 0.0625 %, and 0.015626 % concentrations (unpublished data).

Animal model

The experimental conditions were in compliance with the requirements of the European rules of ethical standards of animal treatment and welfare. Hence, our experiment was approved by the Ethics Committee of the Faculty of Medicine of the Šafárik University in Košice and by the State Veterinary and Food Administration of the Slovak Republic.

Male Sprague-Dawley rats (n=12; 8-10 months of age) were used for experiments and allocated into 3 groups (control – untreated; AB-10 % – treated with the high AB concentration, AB-0.00390625 % – treated with the low AB concentration). For general anesthesia a combination of 33 mg/kg ketamine (Calypsol; Richter Gedeon, Budapest, Hungary), 11 mg/kg xylazine (Rometar a.u.v.; Spofa, Prague, Czech Republic) and 5 mg/kg tramadol (Tramadol-K; Krka, Novo Mesto,

Slovenia) was intramuscularly administered to the rats. One round – 1 cm in diameter – full thickness skin wound was performed under aseptic conditions on the back of each rat. All rats were sacrificed by ether inhalation 21 days after surgery.

Wound treatment

During the treatment all rats were restrained individually in a Plexiglas cage with a circular opening over the wound. In the control group, the aqueous AB extract was not applied. In the experimental groups, the extract was topically applied (by means of an eye dropper) three times a day during the first three days of healing. New extract was prepared each day of wound treatment.

Basic histology and immunohistochemistry

The first half of skin-wound specimens was processed routinely for light microscopy, i.e. fixation in 4 % buffered formaldehyde, dehydration, embedding, cutting, and staining with hematoxylin-eosin.

The second half of wound specimens was cryoprotected by Tissue-Tek (Sakura, Zoeterwoude, Netherlands) and deeply frozen in liquid nitrogen. Cryocut sections (Reichert-Jung, Vienna, Austria) were first mounted on the surface of poly-L-lysine-treated glasses (Sigma-Aldrich, St Louis, MO, USA), then fixed in 2 % (w/v) paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2). Any binding of the used secondary-step antibody preparations was precluded by pre-incubation with normal swine serum (DAKO, Glostrup, Denmark) diluted in PBS for 30 min. Both first- and second-step antibodies were diluted as recommended by supplier, the sources given in Table 1. The specificity of immunohistochemical reactions was verified by the replacement of used antibody by an irrelevant antibody and/or by the omission of the first-step antibody. The lack of cross-reactivity of our homemade anti-galectin-1 antibody was ascertained, if required, after affinity depletion on resin with conjugated galectins (Kaltner *et al.* 2002, Saal *et al.* 2005).

The nuclei of cells were counterstained with the DAPI fluorochrome (Sigma-Aldrich, St Louis, MO, USA), specifically recognizing DNA. The specimens were mounted to Vectashield (Vector Laboratories, Burlingame, CA, USA).

Wound area measurement

The area of healing wounds was measured from standardized photographs as follows. Wounds were photographed with a scale immediately after surgery and at 21 using an Olympus E330 digital camera equipped with a digital ED 50 mm f 2.0 macro objective and a ring set flash SRF-11 (Olympus, Tokyo, Japan). The wound area was then measured on the images using Quick Photo Micro 2.2 software (Premiere, Prague, Czech Republic) and expressed as a percentage of the original wound area created on the day of surgery.

Isolation and in vitro cultivation of human dermal fibroblasts (HDF)

Fibroblasts were isolated from residual skin samples. They were obtained from the Department of Aesthetic Surgery of the Third Faculty of Medicine of Charles University according to the criteria of the Helsinki Declaration with informed consent of patients and approved by local Ethical Committee. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Biochrom, Berlin, Germany) supplemented with 10 % fetal bovine serum (FBS; Biochrom) and antibiotics (streptomycin and penicillin; Biochrom).

Cells were seeded on glass coverslips at a density of 3 000 cells/cm^2 and cultured for 24 hours. Medium containing the tested concentration of AB extract was then added to the cells, which were cultured thereafter for three days.

In vitro cultivation of HaCaT

The HaCaT (human keratinocytes cell line) cell line was obtained from Cell Lines Service (Eppelheim, Germany) (Boukamp *et al.* 1988). Cells were cultured in the Dulbecco's Modified Eagle's Medium (DMEM; Biochrom) supplemented with 10 % FBS (Biochrom) and antibiotics (streptomycin and penicillin; Biochrom).

Cells were seeded on a glass coverslips at a density of 5 000 cells/cm^2 and cultured for 24 hours. Medium containing the tested concentration of AB extract was then added to cells, which were cultured thereafter for four days (medium was changed 1 x during the experiment).

Immunocytochemistry of in vitro cultured keratinocytes and fibroblasts

The adherent cells on the coverslips were washed in PBS and fixed briefly with 2 % (w/v) paraformaldehyde diluted in PBS (pH=7.2). Any binding of the used second-step antibody was precluded by pre-incubation with normal swine serum (DAKO, Glostrup,

Table 1. Reagents for immuno-histo/cyto-chemistry.

Primary antibody	Host	Produced by	Secondary antibody	Produced by	Channel	Stained in
α-smooth muscle actin	mouse monoclonal	DakoCytomation, Glostrup, Denmark	goat anti-mouse	Sigma-Aldrich, St Louis, MO, USA	TRITC-red	HDF, wound
Ki67	mouse monoclonal	DakoCytomation, Glostrup, Denmark	goat anti-mouse	Sigma-Aldrich, St Louis, MO, USA	TRITC-red	HaCaT
Keratin 19	mouse monoclonal	DakoCytomation, Glostrup, Denmark	goat anti-mouse	Sigma-Aldrich, St Louis, MO, USA	TRITC-red	HaCaT
Vimentin	mouse monoclonal	DakoCytomation, Glostrup, Denmark	goat anti-mouse	Sigma-Aldrich, St Louis, MO, USA	TRITC-red	HDF, wound
Collagen-3	mouse monoclonal	Sigma-Aldrich, St Louis, MO, USA	goat anti-mouse	Sigma-Aldrich, St Louis, MO, USA	TRITC-red	wound
Fibronectin	rabbit polyclonal	Dakopatts, Glostrup, Denmark	swine anti-rabbit	Santa Cruz Biotechnology, Santa Cruz, CA, USA	FITC-green	HDF, wound
Galectin-1	rabbit polyclonal	house-made, Gabius laboratory	swine anti-rabbit	Santa Cruz Biotechnology, Santa Cruz, CA, USA	FITC-green	HDF, wound
Wide spectrum cytokeratin	rabbit polyclonal	Abcam, Cambridge Science, Cambridge, UK	swine anti-rabbit	Santa Cruz Biotechnology, Santa Cruz, CA, USA	FITC-green	wound

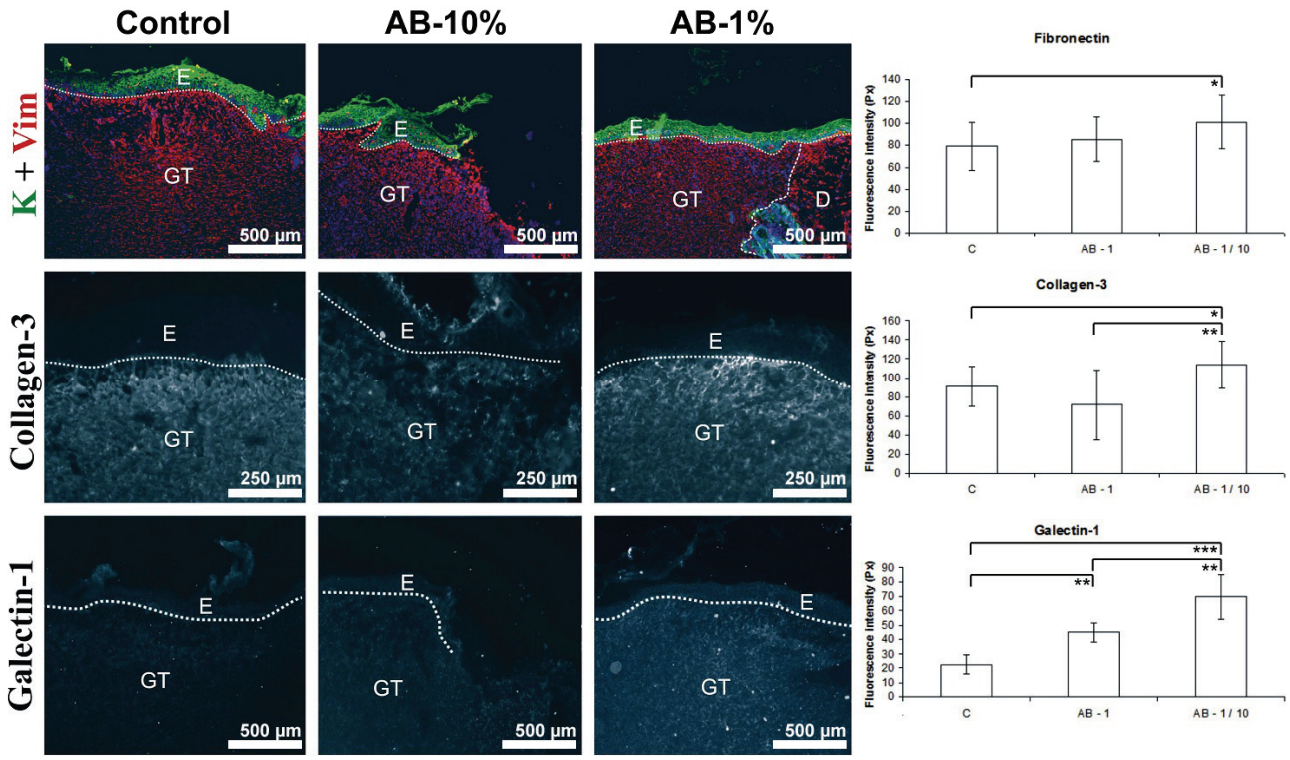

Fig. 1. Control/untreated wounds and wounds treated with two different concentrations of *Atropa belladonna* (AB); **first vertical panel**: completed process of epidermis (E) regeneration and well-formed granulation tissue (GT) rich in collagen-3, but poor in galectin-1; **second vertical panel**: incomplete process or re-epithelialization, granulation tissue poor in collagen-3, but increased content of galectin-1; **third vertical panel**: finished re-epithelialization, well-formed granulation tissue rich in collagen and galectin-1 (D – dermis); fluoresecence for fibronectin intensity is shown in the top graph, collagen-3 fluoresecence intensity in the middle graph, and galectin-1-dependent fluorescence intensity in the bottom graph (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Denmark) diluted in PBS for 30 min. Both the primary and secondary antibodies were diluted as recommended by supplier and are described in Table 1. Their specificity was tested by replacement of a distinct antibody by another polyclonal or monoclonal antibody of the same animal and isotype, but against antigens not present in studied cells. The nuclei of cells were routinely counterstained with DAPI (Sigma-Aldrich). The specimens were mounted with Vectashield (Vector Laboratories).

Fluorescence intensities measurement, cell counting, and image analysis

Both skin sections and coverslips containing cultured cells were analyzed by fluorescence microscopy using a Nikon Eclipse 90i apparatus (Nikon, Tokyo, Japan) equipped with filter-blocks specific for FITC, TRITC and DAPI, respectively, a high-resolution CCD camera Cool-1300Q (Vosskühler, Osnabrück, Germany) and a LUCIA 5.1 computer-assisted image analysis system (Laboratory Imaging, Prague, Czech Republic). Fluorescence intensity was measured under standardized conditions (Klíma *et al.* 2009) using the software given above.

By evaluating Ki67 expression all cells were counted in three visualization field of one coverslip following by counting the Ki67 positive cells. The proliferation activity was than expressed as the percentage of Ki67 positive cells to total no. of cells.

Statistical analysis

Data from the measurement of fluorescence intensities, wound areas, and cell counting were compared by one-way ANOVA followed by Tukey-Kramer post-hoc test. Significance was accepted at $p<0.05$.

Results

Wound histology

At the period of 21 days post surgery regeneration of the epidermis was completely finished in the control and AB-1 % groups, but not in the AB-10 % group (Fig. 1). Only a limited number of luminized

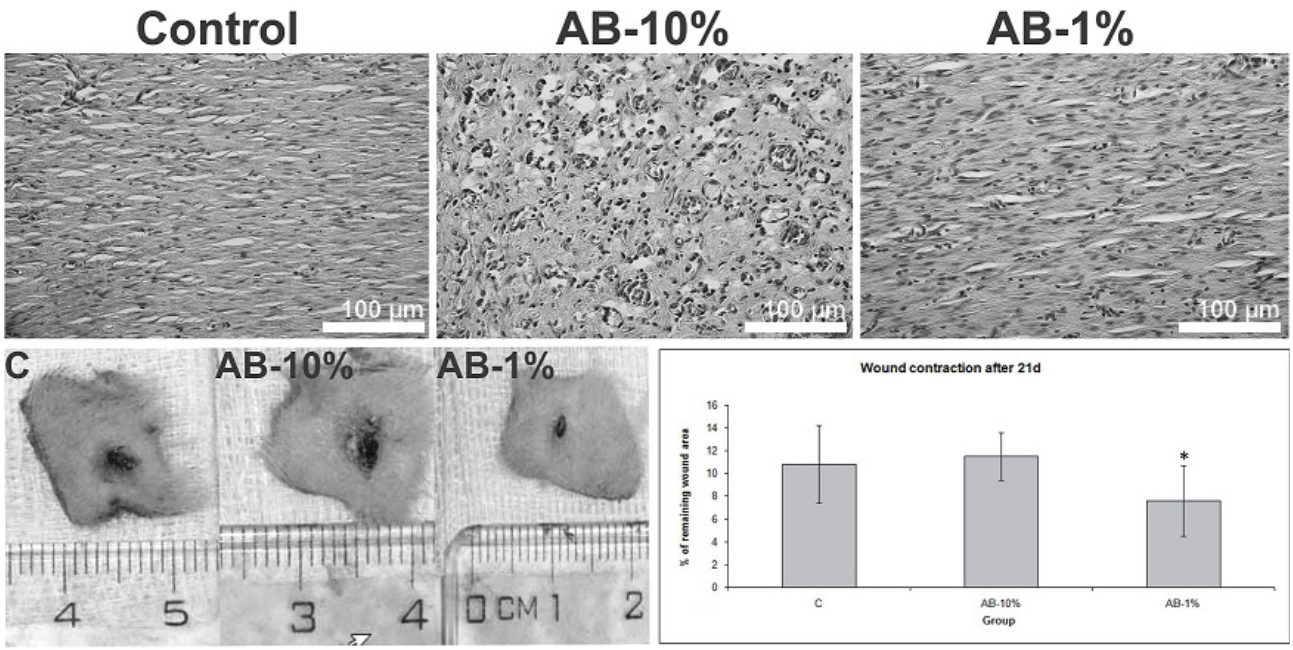

Fig. 2. Effect of *Atropa belladonna* water extract on granulation tissue formation (HE staining) and skin wound closure in rats. Immature granulation tissue formation with high number of luminised vessels in wounds treated with the higher tested AB concentration (AB-10 %). In contrast, control wounds showed normal process of tissue scaring. The most mature scare was observed in wounds treated with the lower tested AB concentration (AB-1 %) where the extract also significantly increased wound contraction in comparison to other groups (* $p<0.05$).

vessels was present in the granulation tissues of control and treated wounds (not shown). The level of fibronectin presence in the granulation tissue was comparable in untreated wounds (control) and wounds treated with the undiluted AB extract (AB-10 %) (not shown). However, treatment of wounds with the diluted AB extract (AB-1 %) resulted in significantly increased production of fibronectin (Fig. 1). Interestingly, granulation tissue of the treated wounds contained a significantly increased amount of galectin-1 when compared to the control (Fig. 1). Of note, the presence of collagen-3 was enhanced in wounds treated with the diluted AB extract, while high extract concentration decreased collagen deposition when compared to the other two groups (Fig. 1).

Wound contraction

Measuring the extent of wound contraction revealed significant effect of lower tested AB concentration (AB-1 %) when compared to control (Fig. 2), while the higher tested AB concentration (AB-10 %) did not significantly modulated wound closure.

HDF

The presence of AB in the culture medium led to the formation of a ECM scaffold which consisted of fibronectin and galectin-1 (Fig. 2). The most prominent newly synthesized ECM network was observed on the coverslips with cells exposed to the higher tested extract concentration (AB-1 %). ECM production was then leveling off in a concentration dependent manner (AB-0.00390625 %).

The presence of AB in the culture medium did not stimulate any transition of fibroblasts into myofibroblasts (not shown). As consequence, no or only a very limited number (up to one per visualization field) of myofibroblasts was present.

HaCaT

All cells expressed wide-spectrum keratin. In contrast to ECM production the most prominent expression of Ki67 was observed in cells exposed to the lower tested AB concentrations (AB-0.00390625 %). With increasing plant concentration Ki67 expression decreased (Fig. 3). No differences were observed in keratin-19 expression between control and AB stimulated cells (not shown).

Discussion

In our previous investigation we found keratinocyte, cultured on 3T3 feeder fibroblasts,

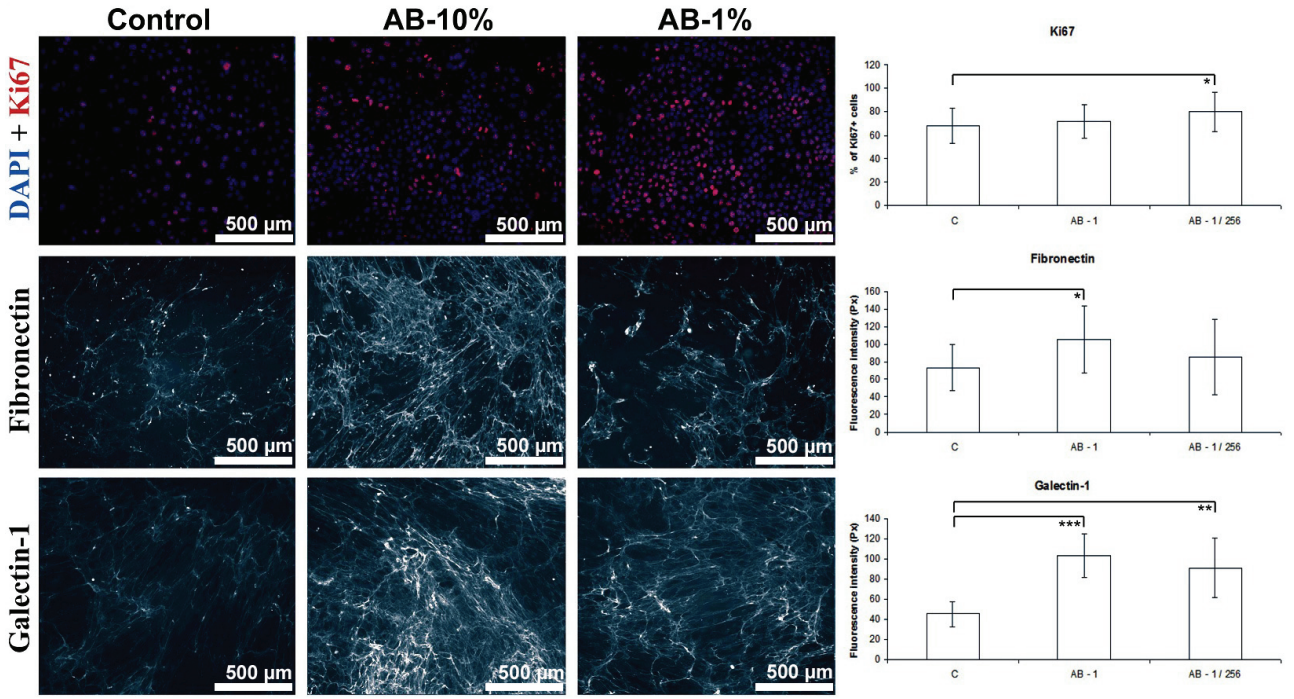

Fig. 3. Control cultures and cultures with cells exposed to two different concentrations of *Atropa belladonna* (AB); **first horizontal panel**: HaCaT keratinocytes with the largest population of Ki67-positive cells in cultures exposed to the lower tested AB concentration; **second and third horizontal panels**: human dermal fibroblast with the highest extent of ECM formation monitored by detection of fibronectin and galectin-1 in cultures exposed to the higher tested AB concentration; the percentage of Ki67-positive cells is shown in the top graph, fibronectin-dependent fluorescence intensity in the middle graph, and galectin-1-dependent fluorescence intensity in the bottom graph (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

expressing keratin-19 in cultures which were supplemented with the highest tested concentration of AB (Gál *et al.* 2009). Such a cell phenotype is evocative of stem-like features (Dvořánková *et al.* 2005), implying a beneficial role for wound healing. In further study we have also shown that keratinocytes cultured on substratum, rich in fibronectin and galectin-1, induced by several galectins, have a poorly differentiated phenotype with keratin-19 expression (Dvořánková *et al.* 2011). In the current investigation, we used the HaCaT cell line and revealed that AB increases Ki67 expression, but did not affect keratin-19 expression. Obviously, previously observed keratin-19 expression may depend on 3T3 feeder fibroblasts which might be affected by AB. Along this line, it was found that wound treatment with *Datura alba* L. (*Solanaceae*) extract led to accelerated epidermis regeneration and increased fibroblast proliferation (Priya *et al.* 2002).

Following AB treatment we previously described increased wound tensile strength and presence of collagen in healing skin wounds (Gál *et al.* 2009). Formation and reorganization of the ECM are crucial processes involved in ensuring sufficient wound stiffness and successful healing. It is well known that fibronectin plays an important role herein. Fibroblasts as a component of granulation tissue secrete both proteases, which are able to proteolytically process surrounding ECM, and new constituents of the ECM including fibronectin and collagen that establish the insoluble matrix (Kumar *et al.* 2003). Therefore, we have monitored fibronectin production after cells and/or wounds were exposed to aqueous AB extract. We found that this treatment of wounds/cells enhanced fibronectin production. Of note, the effect of AB on collagen-3 expression revealed a concentration-dependent manner (AB-10 % decreased, whereas AB-1 % increased collagen deposition in wounds).

Interestingly, newly formed ECM was also rich in galectin-1. This lectin can directly affect cytokine production such as induced IL-10 secretion and decreased IFN-γ production in activated T cells (Stowell *et al.* 2008) and is a potent effector in T cell communication (Wang *et al.* 2009) and its expression is elevated during the inflammatory phase of healing (Gál *et al.* 2011). In addition, pharmacological treatment with galectin-1 inhibited leukocyte recruitment into the peritoneal cavity (Gil *et al.* 2006). Reduction of the inflammatory process by means of lower number of infiltrated PMNLs, also

seen for CD45-positive lymphocytes (Saussez *et al*. 2009), and scarce degranulated mast cells were observed when galectin-1 was administered locally (Rabinovich *et al.* 2000). Obviously, the lectin intimately partakes in the regulation of the inflammatory process (Schwartz-Albiez 2009). The results with experimentally applied galectin-1 or upon its *in situ* up-regulation in tumors can have relevant results for us, in which AB significantly reduced the process of inflammation (Gál *et al.* 2009), a process associated with increased production of galectin-1. Moreover, anti-inflammatory properties of AB were documented by significantly increased wound tensile strength in both treated groups just with unimpressive treatment duration differences (Gál *et al.* 2009). From this point of view, anti-inflammatory effects of AB extract may engage enhanced generation of galectin-1.

Based on our previous (Gál *et al.* 2009) and current investigations we may conclude that the plant extract's effect is probably based on the acceleration of several processes occurring during wound healing. In particular, AB is able to stimulate ECM production, endothelial cells proliferation (as previously shown in HUVECs and may indicate accelerated angiogenesis), and has anti-inflammatory effects (as previously shown in rats). In our previous study, detectable concentrations of tropane alkaloids in water extract of leaves of *Atropa belladonna* L. were confirmed (Gál *et al.* 2009). Concentration of atropine has been more than ten times higher when compared to scopolamine. This is in accordance with results of Zárate *et al.* (1997) which found in leaves of *Atropa beatica* higher amount of atropine than scopolamine, with atropine/scopolamine ratio similar to results obtained in our previous study. Of note the pharmacological effect on wound healing may; however, be modulated either positively or negatively by other additional ballast compounds of the extract. Therefore, the main limitation of presented study is in omission of biological active compounds isolation following their wound healing effect evaluation. Accordingly, further research is needed to explain the underlying mechanisms of action and find optimal therapeutic concentration for any use in clinical practice. But the presented data indicate the potential for further study of this extract, also in defining the possible regulatory pathways.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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