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Wound healing properties of jojoba liquid wax: An in vitro study

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ABSTRACT

Aim of the study: The wound healing properties of jojoba (*Simmondsia chinensis*) liquid wax (JLW) were studied in vitro on HaCaT keratinocytes and human dermal fibroblasts, which are involved in wounded skin repair.
Materials and methods: JLW cytotoxicity was evaluated by the crystal violet staining and the neutral red uptake endpoint. Induction of wound healing by JLW was assessed by scratch wound assay on cell monolayers. The involvement of signaling pathways was evaluated by the use of the Ca^{2+} chelator BAPTA and of kinase inhibitors, and by Western blot analysis of cell lysates using anti-phospho antibodies. Collagen and gelatinase secretion by cells were assayed by in-cell ELISA and zymography analysis, respectively.
Results: Cytotoxicity assays showed that the toxic effects of JLW to these cells are extremely low. Scratch wound experiments showed that JLW notably accelerates the wound closure of both keratinocytes and fibroblasts. The use of inhibitors and Western blot revealed that the mechanism of action of JLW is strictly Ca^{2+} dependent and requires the involvement of the PI3K–Akt–mTOR pathway and of the p38 and ERK1/2 MAPKs. In addition, JLW was found to stimulate collagen I synthesis in fibroblasts, while no effect was detected on the secretion of MMP-2 and MMP-9 gelatinases by HaCaT or fibroblasts.
Conclusions: Taken together, data provide a pharmacological characterization of JLW properties on skin cells and suggest that it could be used in the treatment of wounds in clinical settings.

1. Introduction

Jojoba (*Simmodsia chinensis* (Link) C.K. Schneid) is a perennial woody shrub native to the semiarid regions of southern Arizona, southern California and northwestern Mexico. Jojoba seeds contain a light-gold liquid wax ester, chemically similar to sperm whale's spermaceti, which is the primary storage lipid of the plant (Naqvi and Ting, 1990; Van Boven et al., 1997). Jojoba liquid wax (JLW, also known as jojoba oil) makes up 50% of the seed's dry weight, and as much as 97% of it consists of a mixture of esters of long chain fatty alcohols and long chain fatty acids (Miwa, 1984; Yaron et al., 1982). A non-saponifiable fraction, mainly composed of phytosterols, has also been characterized (Van Boven et al., 1997). The physical properties of JLW are high viscosity, high flash and fire point, high dielectric constant, high oxidative stability and low volatility (Wisniak, 1977). Owing to its peculiar chemical composition and physical properties, the wax is a versatile product having various industrial, culinary and medicinal uses.

JLW is a non-irritating, non-comedogenic product (Johnson, 1992), which is currently used in a number of skin care products, mainly as a moisturizer, as well as in hair conditioners. However, both traditional uses and research studies testify that JLW has medicinal properties and can be used as a remedy, mainly for skin disorders. Spanish explorers reported that the native Americans of the Sonora desert extracted JLW from the seeds for various cosmetic and medicinal purposes, such as treating sores and wounds (Bloomfield, 1985).

JLW has been also used in folk remedies for renal colics, headache, sunburn, chaffed skin, and hair loss (Yaron, 1987). Dermatological research has shown that JLW may help to reduce inflammation (Habashy et al., 2005) and is effective in the treatment of acne and psoriasis (Mosovich, 1985).

Severe wounds and chronic ulcers are a major concern in the medical field. Chronic wounds affect a large number of patients and current worldwide estimates suggest that nearly 6 million people suffer from this kind of disorders. Wound healing is a complex process coordinating a variety of cellular activities such as migration to the wounded area, proliferation, and deposition and remodelling of extracellular matrix, mainly the collagen lattice (Martin, 1997). A plethora of materials for wound dressing, skin substitutes, and recombinant growth factors have been shown to enhance the healing process, and some of them have been introduced into the clinical setting with therapeutic efficacy (Ovington, 2007). Many

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natural products are also known to possess wound healing properties, based on both anedoctal and scientific evidence (Fatima et al., 2008; Lindblad, 2008; Davis and Perez, 2009). Hence, there is a great interest to find new wound healing products and to characterize their mechanism of action.

Given the wide cosmetic use and the dermatological properties of JLW, this study was designed to explore the potential wound healing effects of this plant product. We used a well assessed in vitro scratch wound healing model consisting of human fibroblasts and keratinocytes (Ranzato et al., 2008, 2009a; Burlando et al., 2009). In vitro tests are now widely employed in pharmacological research because of ethical reasons and of their usefulness in bioactive-guided fractionation and determination of active compounds (Houghton et al., 2005).

Given the overlapping phases of the wound healing process, a plant-based remedy should affect at least two different processes before it can be said to have some scientific support for wound healing use. For this reason, we included in our experiments two cell types that play main roles in wounded skin repair. Fibroblasts are attracted into the site of wound to initiate the proliferative phase of repair and matrix deposition, while keratinocytes are involved in the re-epithelialization phase (Gurtner et al., 2008). Our data showed that JLW notably accelerates wound closure by acting both on fibroblasts and keratinocytes. The process resulted to be strictly Ca^{2+} dependent and to require the involvement of p38, ERK1/2, and the PI3K–Akt–mTOR pathway. JLW also showed the ability of inducing collagen I synthesis in fibroblasts. Taken together, these data suggest the pharmaceutical implementation of JLW as an adjuvant in wound healing therapies.

2. Materials and methods

2.1. Cell culture and reagents

All reagents were from Sigma–Aldrich, unless otherwise indicated. Pure JLW was from Cosmeca srl (Trofarello, Italy). HaCaT cells are immortalized human skin keratinocytes that mimics many properties of normal epidermal keratinocytes, are not invasive, and can differentiate under appropriate experimental conditions (Boukamp et al., 1988). Human primary fibroblasts were obtained from skin biopsies (Ranzato et al., 2009b). Cells were maintained at 37 °C, 5% CO_2, in Dulbecco-modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS, Euroclone, Pero, Italy) and 1% antibiotic mixture.

2.2. Crystal violet assay (CV)

Cell viability was assessed by staining cells with crystal violet dye. Briefly, HaCaT and fibroblasts were seeded on 96-well plates (20,000 cells/ well), grown for 24 h prior to experiments and then exposed to various concentrations of JLW for 48 h. Thereafter, the medium was removed, cells were gently washed with phosphate buffered saline (PBS), stained for 10 min with 0.5% crystal violet in 145 mmol/L NaCl, 0.5% formal saline, 50% ethanol, and washed thrice with water. Crystal violet was eluted from cells with 33% acetic acid and the absorbance of the supernatants was measured at 540 nm in a microplate reader (Sirio S, SEAC, Florence, Italy).

2.3. Neutral red uptake (NRU) assay

The NRU assay is a cell viability test based on the incorporation of neutral red dye into the lysosomes of viable cells after incubation with the test agent. Cells were seeded and treated as described for the CV assay, and then analysed by following the method reported by Borenfreund et al. (1989).

2.4. Scratch wound test

Scratch wound analysis was performed in confluent monolayers of fibroblasts or HaCaT, as described in Ranzato et al. (2008, 2009a).

The width of the wound space was measured at wounding and at the end of treatments, using an inverted Televal microscope (Carl Zeiss Inc., Thornwood, NY) equipped with a digital camera, and the NIH ImageJ software. Wound closure was determined as the difference between wound width at 0 and 6 or 24 h for HaCaT or fibroblasts, respectively.

2.5. In-cell enzyme-linked immunosorbent assay (ELISA)

Collagen I and III production was evaluated by in-cell ELISA. Briefly, cells were seeded on 96-well plates (15,000 cells/well), grown for 24 h prior to experiments and then exposed to various concentrations of JLW for 24 h. The medium was then removed and cells were washed once with PBS, fixed for 10 min with 4% formaldehyde, washed thrice with wash buffer (1× PBS, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% Triton) followed by a blocking step of 30 min with 3% BSA in wash buffer. Aliquots of 100 μL of mouse monoclonal abs against cow collagen type I (Cat. No. b6308, Abcam, Cambridge, UK), or human collagen type III (Cat. No. ab6310, Abcam), each of them diluted 1:300 in wash buffer containing 1% BSA, were added to wells and incubated for 2 h at room temperature under mild agitation. After this step, and after each of the following ones, the plates were emptied and repeatedly washed with wash buffer, to remove the excess of reagents. Aliquots of 100 μL of horseradish peroxidase-labelled secondary antibody (Bethyl Laboratory, Montgomery, TX, USA), diluted 1:1000 in PBS, were added to wells for 1 h at room temperature under mild agitation. Aliquots of 50 μL of freshly prepared peroxidase substrate system for ELISA (3,3′,5,5′ tetramethylbenzidine, TMB) were added to microplates, and after 5 min incubation the reaction products were read at 620 nm in the microplate reader.

2.6. Gelatin zymography

HaCaT and fibroblasts were grown in 12-well plates to subconfluence and then treated for 24 h with JLW as indicated. During treatments, the concentration of FBS in the culture medium was reduced to 0.5% in order to avoid albumin interference in zymograms. Conditioned media were then collected, spinned and stored at −20 °C until use. The corresponding cells were lysed with a NP-40 buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40, and Sigma–Aldrich Protease Inhibitors Cocktail). Total protein present in cell lysates was determined using the bicinchoninic acid-based BCA Protein Assay Kit (Pierce, Rockford, IL, USA), following the manufacturer's protocol. Media were mixed with an equal volume of a 2× loading buffer (0.4 M Tris HCl, pH 6.8, 20% glycerol, 5% SDS, and a pinch of bromophenol blue) and then loaded onto a non-denaturing, 8% polyacrylamide gel containing 0.1% (w/v) gelatin. The volume of each loaded sample was adjusted according to the protein concentration in the corresponding cell lysate. The proteins of cell media were then resolved by electrophoretic run at 150 V for 90 min, and thereafter the SDS in the gel was removed by incubation with 2.5% Triton X-100 in PBS for 1 h. Gelatinolytic activity was developed in a buffer containing 5 mM $CaCl_2$, 200 mM NaCl, 1 μM $ZnCl_2$, and 50 mM Tris, pH 7.5, by incubation for 18 h at 37 °C. The enzymatic activity was then visualized by staining the gel in 0.5% Coomassie Brilliant Blue G-250, 10% glacial acetic acid, 10% ethanol, followed by destaining in 30% methanol and 10% acetic acid. Gels were acquired with a GS-710 densitometer, digitized by the Quantity One Image Software (Bio-Rad, Hercules, CA), and analysed using the ScionImage 4.0.3.2 software (Scion Co., USA).

Table 1
Median effective concentrations (EC_{50}) derived from cytotoxicity tests after exposure of skin cells to JLW or SDS for 48 h.

		JLW	SDS
HaCaT	CV	21 (19–23)	0.006 (0.006–0.007)
	NRU	18 (15–21)	0.007 (0.006–0.008)
Fibroblasts	CV	21 (18–24)	0.012 (0.011–0.012)
	NRU	29 (26–32)	0.012 (0.008–0.02)

CV: crystal violet assay. NRU: Neutral red uptake assay. Data are expressed as percent concentration (v/v). 95% confidence limits are given in parentheses.

Table 2
Minimum effective concentrations (EC_{05}) derived from cytotoxicity tests after exposure of skin cells to JLW or SDS for 48 h.

		JLW	SDS
HaCaT	CV	3.8 (2.9–4.8)	0.002 (0.002–0.003)
	NRU	2.5 (1.3–4.3)	0.001 (0.0006–0.002)
Fibroblasts	CV	3.0 (1.7–4.9)	0.009 (0.008–0.01)
	NRU	10 (7.7–13)	0.010 (0.010–0.011)

Data are expressed as in Table 1.

2.7. Western blotting

Cells were grown in 12-well plates to subconfluence, exposed to JLW at the indicated concentrations for 24 h, and then lysed in Laemmli buffer. Thereafter, 40 μg of protein from cell lysates were loaded on gel, subjected to SDS-PAGE (12% polyacrylamide gel), and transferred onto nitrocellulose membranes using a Bio-Rad Mini Trans Blot electrophoretic transfer unit. Membranes were blocked for nonspecific protein with 5% nonfat dry milk in PBS and then probed for 1 h at room temperature with specific primary antibodies against p-ERK1/2, p-p38, p-Akt, p-S6, ERK1/2, p38, Akt, and b-actin (1:1000, Cell Signaling Technology, Celbio SpA, Milan, Italy). Membranes were then washed three times (10 min per wash) with PBS additioned with 0.05% Tween-20 to remove unbound antibodies, and then further incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000). Membranes were developed by the ECL kit (Millipore, Billerica, MA, USA), according to the manufacturer's protocol, acquired with a ChemiDoc XRS (Bio-Rad Laboratories, Hercules, CA), digitized with Quantity One software (Bio-Rad Laboratories), and normalized against proper loading controls.

2.8. Statistics

Data were analysed by ANOVA and the post hoc Tukey's test, using the Instat software package (GraphPad Software Inc, San Diego, CA). Median (EC50) and minimum (EC05) effective concentrations and their 95% confidence intervals were determined by using a downhill logistic dose-response curve.

3. Results

3.1. Cell proliferation and metabolism

In order to achieve an evaluation of JLW irritating power, we performed cytotoxicity tests on skin cells by comparing JLW to the model irritant sodium dodecyl sulfate (SDS). Cells were exposed to increasing concentrations of JLW (1–60%, v/v) or SDS for 48 h and then analysed by the CV and NRU assays. JLW concentrations higher than 10% were achieved by pre-mixing with FBS followed by sonication. Data showed that JLW has an extremely low toxicity on both cell types (Tables 1 and 2), in agreement with previous observations (Johnson, 1992).

3.2. Scratch wound repair of HaCaT

Confluent monolayers of HaCaT were scratch wounded as described in the Methods and then allowed to re-epithelialize for 6 h at 37 °C in the presence or absence of JLW at the indicated concentrations. One series of samples, used as positive controls, were exposed to a dose of 20% (v/v) of a platelet lysate (PL), which had been previously shown to promote scratch wound healing in these cells (Ranzato et al., 2009b and references therein). The PL was obtained from blood samples as described in Ranzato et al. (2008). Cells exposed to JLW showed significantly higher wound closure rates at 6 h with respect to controls ($p < 0.01$). The strongest effect, obtained with 0.5% JLW, was not statistically different from that of PL (Fig. 1).

To investigate the mechanism of action of JLW on wound closure, we performed scratch wound experiments using PD98059 (ERK inhibitor, 10 μM), SB203580 (p38 inhibitor, 20 μM), wort-

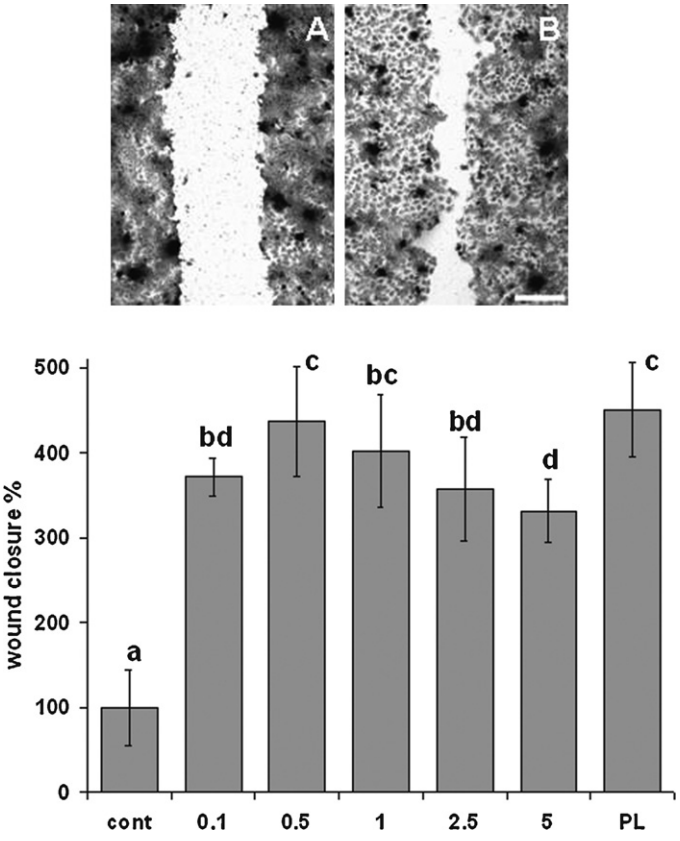

Fig. 1. Scratch wound healing of HaCaT confluent monolayers. Cells cultured in 12-well plates were mechanically scratched with a sterile 0.1–10 μL pipet tip and then allowed to re-epithelialize for 6 h at 37 °C in the presence of JLW at the indicated concentrations (%, v/v). One sample was exposed to 20% platelet lysate (PL) as positive control (see text). Upper panel: micrographs of scratch wounded HaCaT monolayers incubated under control conditions (A) or in the presence of 0.5% JLW (B), and then stained with blue toluidine and observed 6 h after wounding. Scale bar, 200 μm. Lower panel: measurements of wound closure rates expressed as the difference between wound width at 0 h and 6 h. Each bar represents mean ± SD of two independent experiments, each with $n = 20$. The mean of controls was set at 100%. Different letters on bars indicate groups significantly different from each other according to the Tukey's test ($p < 0.01$).

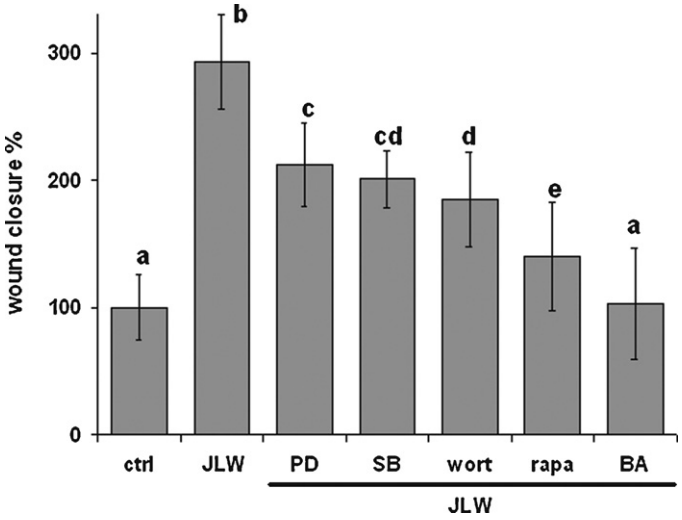

Fig. 2. Effect of different inhibitors on scratch wound repair of HaCaT monolayers. Data were recorded at 6 h after wounding of cells exposed or not to 0.5% JLW, in the presence or absence of various inhibitors. Data and statistics as in Fig. 1. PD: PD98059; SB: SB203580; wort: wortmannin; rapa: rapamycin; BA: BAPTA-AM.

mannin (PI3K inhibitor, 500 nM), rapamycin (mTOR inhibitor, 100 nM) and the cell-permeant calcium chelator BAPTA-AM (30 μM). Confluent cells were scratched in the absence or presence of each inhibitor, with or without 0.5% JLW, and wound closure was then measured at 6 h post wounding. The inhibitors did not alter the basal wound closure rate with respect to control ($p > 0.05$), with the exception of BAPTA ($p < 0.01$). Conversely, the increase of wound closure rate induced by JLW was inhibited to various extents by kinase inhibitors, in this order: rapamycin > wortmannin ≥ SB203580 ≥ PD98059. However, the strongest inhibition was obtained with BAPTA, which completely abolished the effect of JLW (Fig. 2). The vehicle alone (0.1% DMSO) produced no wound healing variation with respect to control, either in the presence or absence of JLW ($p > 0.05$).

3.3. Scratch wound repair of fibroblasts

Human dermal fibroblast monolayers were cultured in 12-well plates, mechanically scratched and exposed to JLW for 24 h. One series of samples were exposed to PL as positive control. Cells exposed to JLW showed significantly higher wound closure rates at 24 h with respect to controls ($p < 0.01$). Moreover, the wound closure rates induced by 0.5% and 1% JLW were not statistically different from the effect of PL (Fig. 3). The use of the above drugs showed variable inhibitory effects on the wound closure rate induced by JLW, ranking in the following order: rapamycin ≥ wortmannin ≥ PD98059 ≥ SB203580. The strongest inhibitory effect of rapamycin completely abolished the JLW-induced wound healing (Fig. 4).

3.4. Protein phosphorylation signaling

In order to explore the role of phosphorylation cascades in the mechanism of action of JLW, we examined by Western blotting the activation of the mTOR downstream ribosomal peptide S6, the PI3K-dependent kinase Akt, and the p38 and ERK1/2 MAP kinases at 6 h post wounding. These data showed that JLW induces in HaCaT and fibroblasts the activation of all these pathways in a dose-dependent manner (Fig. 5), hence confirming their involvement in JLW-driven wound healing, as suggested above by the use of specific inhibitors.

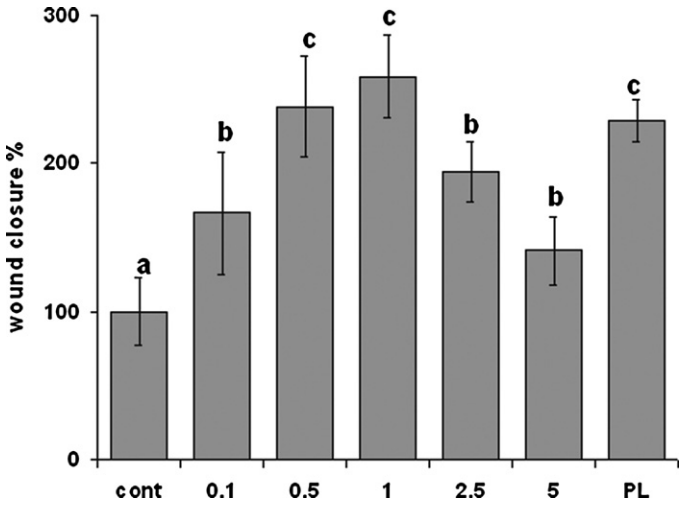

Fig. 3. Scratch wound healing of human dermal fibroblast monolayers. Cells were cultured in 12-well plates, mechanically scratched as described above, and exposed to JLW at the indicated concentrations for 24 h. One sample was exposed to PL as in Fig. 1. Data and statistics as in Fig. 1.

3.5. Collagen synthesis

Data from in-cell ELISA showed that JLW has the ability of inducing the synthesis of collagen type I in fibroblasts (Fig. 6). Also in this case, the maximum effect was found at a concentration of 0.5% JLW. A slight, not significant induction of collagen synthesis was observed in HaCaT at a concentration of 0.01% JLW. Conversely, no effect was observed on collagen type III expression in both fibroblasts and HaCaT (not shown).

3.6. Matrix metalloproteinase 2 (MMP-2) and MMP-9 secretion

To investigate the effect of JLW on the secretion of MMP-2 and MMP-9 (72-kDa and 92-kDa type IV collagenases, respectively) HaCaT and fibroblast conditioned media were collected from cultures grown in 0.5% FBS, following 24 h incubation with JLW. The MMP-9 and MMP-2 present in the conditioned medium of the two cell types were detected by gelatin zymography and identified by comparison with homologous gelatinases obtained from serum-free medium conditioned by HL60 cells stimulated for 18 h with 100 nM PMA (Fiore et al., 2002) (Fig. 7). Zymography data showed

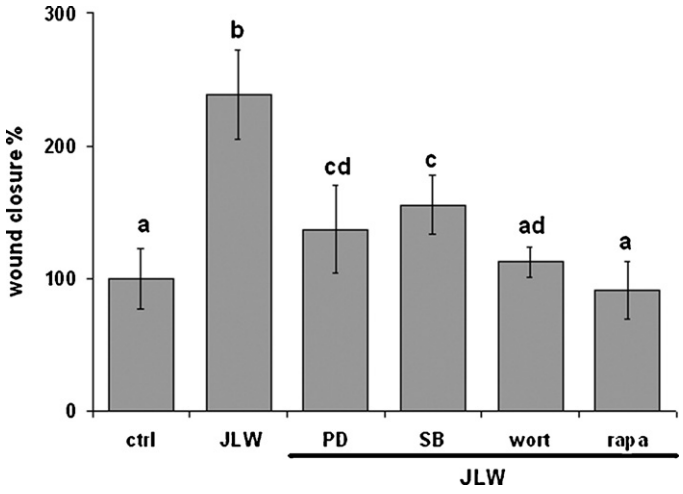

Fig. 4. Effect of different inhibitors on scratch wound repair of dermal fibroblast monolayers. Data were recorded 24 h after wounding of cells exposed or not to 0.5% JLW, in the presence or absence of various inhibitors. Data and statistics as in Fig. 1.

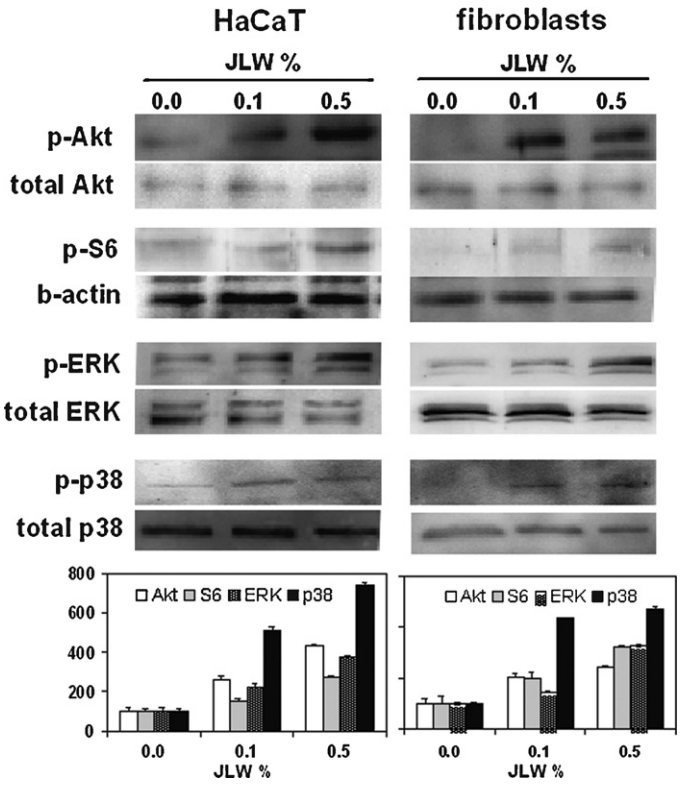

Fig. 5. Effect of 6-h exposures to different doses of JLW on the phosphorylation of Akt, S6, ERK1/2, and p38 in HaCaT and fibroblasts. Upper panel group: total cell lysates (40 μg protein/lane) were separated on 12% SDS-PAGE, transferred to a nitrocellulose membrane, labelled with anti-phospho-Akt, -phospho-S6, -phospho-ERK1/2, or -phospho-p38, and then stripped and reprobed with anti-Akt, -b-actin, -ERK1/2, or -p38, respectively, as internal loading controls. Lower panel group: measures of phosphorylation levels obtained from the ratio between the optical densities of the bands of the phosphorylated form and of the corresponding loading control. Data are means ± SD ($n = 3$). The means of controls (0.0% JLW) have been set to 100%. All groups are significantly different from controls according to the Dunnett's test ($^*p < 0.01$), except for S6 at 0.1% JLW in HaCaT.

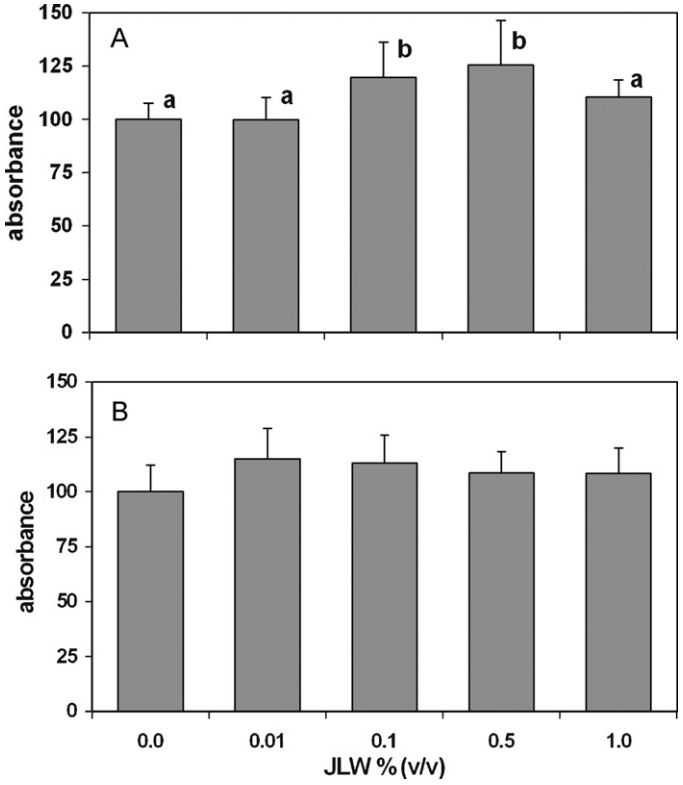

Fig. 6. In-cell ELISA revealing collagen type I production in fibroblasts (A) and HaCaT (B) exposed to increasing concentrations of JLW for 24 h. Data are mean ± SD of % absorbances obtained from two independent experiments with five replicates each. Statistics as in Fig. 1.

that in the HaCaT conditioned medium both MMP-9 and MMP-2 are quite detectable (Fig. 7A), while in fibroblasts MMP-2 is expressed at good levels and MMP-9 is barely detectable (Fig. 7B). These patterns represent the typical constitutive gelatinase expression of these cells types (Fini and Girard, 1990). Moreover, exposure to doses of 0.1 and 0.5% JLW, which were found to strongly increase wound healing, did not induce significant variations in the gelatinase activities of either cell types (Fig. 7).

4. Discussion

Wound healing is of great relevance for skin medicine and a particular focus is set on natural compounds. JLW had never been tested before on wound mechanism, and we decided to perform a set of experiments in order to highlight its effects on a wound healing model.

The JLW treatment showed very low cytotoxic effects on HaCaT and fibroblasts, as demonstrated already with animals (Taguchi and Kunimoto, 1977), suggesting that JLW can be classified as non-toxic substance, and used safely not only for external applications on healthy skin, but also as a dressing on wounds.

Our scratch wound data showed beyond reasonable doubt that JLW induces a marked increase of the wound repair capabilities of fibroblasts and keratinocytes. We compared this effect to the stimulation of a platelet lysate (PL) that is used as a dressing in clinical practice to improve wound healing (Mazzucco et al., 2004). Most interestingly, our data showed that JLW at 0.5% and 1% (v/v) was able to stimulate wound closure to an extent comparable to that of PL.

Hence, in order to get better insight into the JLW-stimulated wound healing, we performed a series of experiments using a battery of cell signal translation inhibitors. The results of these analyses suggest that different pathways are involved in the mechanism of

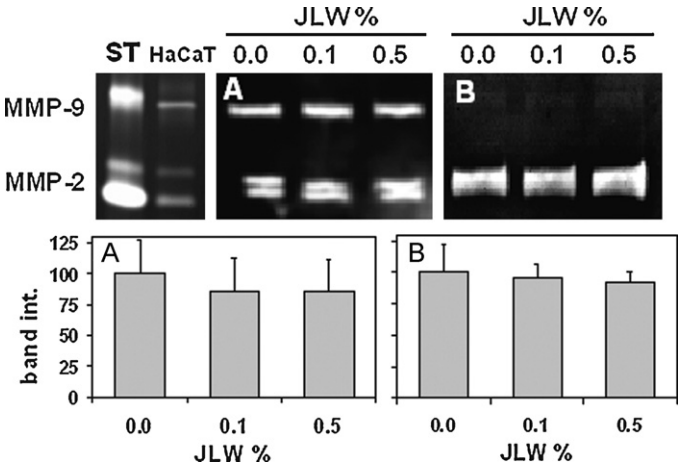

Fig. 7. (Left upper panel) Zymography showing gelatinolytic activities of standard MMP-9 and MMP-2 (ST) obtained from HL60 cells stimulated with 100 nM PMA (see text), and of their homologous gelatinases present in HaCaT conditioned medium. (Center and right upper panels) Zymographies showing the effect of 24-h exposures to different doses of JLW on the activity of MMP-2 and MMP-9 in the cell medium of HaCaT (A) and fibroblasts (B). (Lower panels) Levels of MMP-9 activity in HaCaT medium (A) and of MMP-2 activity in fibroblast medium (B), after treatments carried out as above, quantitated by densitometric image analysis of zymographies, and expressed as mean ± SD ($n = 6$ different experiments). The means of controls (0.0% JLW) have been set to 100%.

action of JLW. In addition, the pattern of inhibition was similar in keratinocytes and fibroblasts, suggesting that JLW acts through the same mechanism in both cell types. Cell calcium seems to play a basic role, as observed in similar studies on these cells, while the use of SB203580 and PD98059 and Western blot analysis provided evidence for the involvement of p38 and ERK1/2, two well known pathways regulating cell migration and wound healing (see e.g. Sharma et al., 2003).

However, a main novelty of this study was the finding that rapamycin was the most active kinase inhibitor in the suppression of JLW-induced wound healing, and that wortmannin was the second most active one. These data strongly suggest that JLW activates the PI3K–Akt–mTOR pathway in both keratinocytes and fibroblasts, as also confirmed by Western blot analysis. Such a pathway seems to represent an integral component of the normal wound healing process, and it has been shown in particular that mTOR activation increases wound healing (Squarize et al., 2010), while its pharmacological inhibition with rapamycin delays wound closure (Buhaescu et al., 2006; Ekici et al., 2007). The role of mTOR in tissue regeneration is further supported by a reported antilymphangiogenic effect of rapamycin (Huber et al., 2007).

Also, mTOR was found to upregulate the expression of collagen and other extracellular matrix proteins (Ong et al., 2007), which is consistent with our observation that JLW mildly stimulates collagen I secretion in fibroblasts. This latter kind of activity could significantly contribute to the suitability of JLW for wound treatment, as the synthesis and deposition of extracellular matrix is a critical feature in the healing of chronic and acute wounds, where matrix defects result in compromised function (Badylak et al., 2010).

mTOR is known to regulate cell growth and survival by integrating nutrient and hormonal signals, and therefore, the rapamycin-sensitive effect of JLW on wound healing suggests a possible role of the wax components as agents able to induce cell responses similar to nutrient stimulation. JLW is mainly composed of long-chain, monounsaturated fatty acids and alcohols (Miwa, 1984), while mTOR is known to be involved in lipid metabolism (Laplante and Sabatini, 2009). Yet, mTOR-dependent connections between fatty acids and cell locomotory activities are poorly documented. However, it is interesting to note that the incubation of hepatoma cells with the monounsaturated oleic acid was found to activate Akt, and increase cell proliferation, migration and invasiveness in a rapamycin-sensitive manner (Vinciguerra et al., 2009). Also, JLW contains campesterol, stigmasterol and sistosterol as main components of its unsaponifiable fraction, and it has been found that beta-sitosterol increases PI3K/Akt activation in tumor cells (Kim et al., 2008). This body of evidence suggests that various components of JLW could produce a series of stimulations converging on the PI3K–Akt–mTOR pathway, and collectively give rise to a rapamycin-sensitive stimulation of wound healing.

Hence, taken together, our data provide some hints arguing that the activation of the PI3K–Akt–mTOR signaling could be a main trait of the JLW mechanism of action. Such a feature could render JLW particularly interesting for a possible synergistic interaction with other medical wound dressings, like platelet derivatives, which have been found to act on different cell types mainly via MAP kinase activation, while PI3K–Akt seems to invariably play a subordinate role (Ranzato et al., 2008, 2009a,b).

The role of gelatinases in wound healing has been repeatedly investigated. Some of these studies have shown that MMP-2 does not seem to be essential for the healing process (Frossing et al., 2010), or that it is involved in undesired effects such as keloid formation by fibroblasts (Imaizumi et al., 2009). Conversely, MMP-9 is believed to play a determinant role in keratinocyte migration and recruitment to wounded sites (Gillard et al., 2004), as also suggested by the finding of strong MMP-9 stimulation in keratinocytes by a wound-healing promoting platelet derivative (Ranzato et al., in press). Hence, given the absence of effects exerted on MMP-9, JLW seems to have the best efficacy in wound healing if combined with other wound-active substances.

In conclusion, our findings provide a scientific characterization of JLW properties on skin cells and suggest that it could be proficiently used in the treatment of wounds in clinical settings. Moreover, the activation of the PI3K–Akt–mTOR signaling is a pharmacologic trait that renders JLW interesting also for other clinical applications.

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