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RESEARCH PAPER

Genistein aglycone improves skin repair in an incisional model of wound healing: a comparison with raloxifene and oestradiol in ovariectomized rats

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Background and purpose: Oestrogen loss at menopause is frequently related to poor wound healing. Genistein has been tested in anti-ageing cosmetic preparations with interesting results on skin health. Here, we investigated the effects of the genistein aglycones, given systemically, in an incisional model of wound healing, compared to systemic oestradiol and raloxifene.

Experimental approach: Six months after ovariectomy (OVX), rats were randomly assigned to groups of 12 animals each and treated daily with genistein aglycone (1 and 10 mg·kg^{-1} s.c.), raloxifene hydrochloride (0.05 and 0.5 mg·kg^{-1} s.c.) or 17-α-ethinyl oestradiol (0.003 and 0.03 mg·kg^{-1} s.c.) for 12 weeks. Untreated OVX and sham OVX rats were used as controls. Then, 14 or 7 days before the end of the experiment, an incisional wound healing procedure was performed and skin specimens were collected to evaluate molecular, histological and functional measurements.

Key results: Seven and fourteen days after wounding, samples from OVX rats showed a decrease in transforming growth factor-β1, tissue transglutaminase 2 and vascular endothelial growth factor compared to samples from sham OVX rats. Oestradiol, raloxifene and genistein all significantly modified this decrease, but the lowest genistein dose exerted a greater effect than the other treatments. Moreover, the lowest dose of genistein was the most effective in improving skin healing and wound tensile strength.

Conclusions and implications: Genistein aglycone might be an alternative therapy for the management of skin wound healing.

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Keywords: wound healing; skin; menopause; rats; genistein aglycone; oestrogen; raloxifene

Abbreviations: ER, oestrogen receptor; HRT, hormone replacement therapy; OVX, ovariectomized; SERMs, selective oestrogen receptor modulators; TG2, tissue transglutaminase 2; TGF-β1, transforming growth factor-β1; VEGF, vascular endothelial growth factor

Introduction

Several experimental and clinical studies showed that oestrogen loss at menopause is frequently followed by skin atrophy, decreased collagen and water content, loss of elasticity and manifestations of hyperandrogenism (Brincat *et al.*, 1985; 1987; Affinito *et al.*, 1999). Further, the cumulative effect of oestrogen deficiency on skin is thought to contribute to poor wound healing that accompanies ageing in humans (Brincat *et al.*, 2005). These negative effects on cutaneous wound healing include an increased susceptibility to trauma, resulting in fragile skin that tears and bruises easily. Overall, the specific role of oestrogens in wound healing and metabolic processes involved in wound repair is poorly understood. Age-related delays in wound healing have been partially

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attributed to low levels of transforming growth factor-β1 (TGF-β1) (Ashcroft *et al*., 1997a), decreased collagen synthesis (Ashcroft and Mills, 2002) and increased presence of elastase (Herrick *et al*., 1997). Accordingly, it has been shown that oestrogens exert a positive influence on healing by inducing TGF-β1 secretion by dermal fibroblasts (Ashcroft *et al*., 1997a). Reduced oestrogen levels have been also associated with impaired cytokine signal transduction in the inflammatory phase of wound repair even if a direct association with oestrogen treatment and the inflammatory phase of wound healing remains to be investigated (Ashcroft and Ashworth, 2003).

Oestrogen treatment has also been shown to increase collagen deposition in wounds of ovariectomized (OVX) rats (Ramamurthy *et al*., 1999), but systemic oestrogen exerts either no effect (Murthy *et al*., 1974; Pirila *et al*., 2001) or decreases collagen deposition (Ashcroft *et al*., 1997b), depending on dose and time since wounding. In general, investigations with animals offer contradictory findings about the effect of oestrogen levels on the stages of wound healing. These discrepancies are likely due to differences in species, duration of treatment and methodologies employed (Calvin, 2000).

Hormone replacement therapy (HRT) is commonly used to treat menopausal symptoms (Castelo-Branco *et al*., 1999; Writing Group for the Women's Health Initiative Investigators, 2002; Ahlborg *et al*., 2004) and appears to significantly accelerate wound healing (Ashcroft *et al*., 1997a). In this context, a randomized, double-blind study in elderly men and women demonstrated that topical oestrogen reduces activity of elastase in skin wounds when compared with placebo (Ashcroft *et al*., 1999). Overall data on wound healing in humans, however, are still very limited, and further work is necessary before conclusive recommendations can be drawn. Indeed, perceptions of oestrogen side effects and risk profiles in some women limit the therapeutic utility of HRT, thus narrowing the possibility of this use in wider populations. Consequently, different alternative therapeutic approaches have to be considered in the last years.

Recently, selective oestrogen receptor modulators (SERMs) were developed in an attempt to achieve the beneficial effects of oestrogen, while minimizing the detrimental side effects in target tissues through specific oestrogen receptor (ER) interaction. Raloxifene is one of the most studied SERMs and has been proven effective in ameliorating wound healing in OVX mice compared to oestradiol (Hardman *et al*., 2008). The phyto-oestrogen genistein has also been considered as a natural SERM and might play a preventive role in impaired wound healing without the harmful oestrogenic side effects on breast and uterine tissue (Cassidy, 2003).

Specifically, genistein aglycone showed consistent efficacy in managing conditions of oestrogen deprivation, and isoflavone-containing cosmetic creams have been used to improve skin dryness and wrinkles (Rona *et al*., 2004). Our research group has already demonstrated in previous experimental and clinical studies the efficacy and safety of genistein aglycone in a low oestrogen environment, and this promising profile may be a direct consequence of greater genistein affinity for the ER-β than ER-α (Squadrito *et al*., 2003; Atteritano *et al*., 2007; Marini *et al*., 2007; 2008a,b; 2009; Bitto *et al*., 2008; 2009a,b; D'Anna *et al*., 2009). Notably, ER-β is more widely distributed within the skin and skin structures (Thornton *et al*., 2003), and, additionally, at physiological concentrations, oestradiol up-regulates ER-β receptors in keratinocytes inducing proliferation (Merlo *et al*., 2009). Oestrogens also target human skin fibroblasts, and because ER-α and ER-β co-express in human skin fibroblasts (Haczynski *et al*., 2002), it is very likely that ER ligands such as genistein could also play a regulatory role on extracellular matrix in the skin.

The present study originated from a parent experiment on OVX rats treated with either genistein aglycone or oestradiol or raloxifene, demonstrating that the isoflavone genistein is able to reverse osteoporosis induced by oestrogen loss (Bitto *et al*., 2008). In these same animals, we produced an incisional wound in order to evaluate the healing properties of a long-term continued therapy with the mentioned compounds. The results of these experiments have helped to clarify the complex molecular interplay between skin and phyto-oestrogens in menopause, and the possibility of preventing the cellular and molecular changes that hamper proper wound healing in post-menopausal women.

Methods

Animals and treatments

All animal care and procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA). The experimental protocols were reviewed and approved by the Ethics Committee of the University of Messina, and have been detailed in the parent study (Bitto *et al*., 2008). During the experiments, the animals were housed in the Animal Facility of the Department of Clinical and Experimental Medicine and Pharmacology of University of Messina, maintained under controlled environmental conditions (12 h light–dark cycle, temperature approximately 24°C), and provided with standard food for laboratory animals and water *ad libitum*.

Briefly, a total of 84 OVX and 12 sham OVX female Sprague–Dawley rats (Charles River, Calco, Italy), aged 12 weeks and weighing about 250–275 g were purchased. After 6 months, the animals were divided into eight groups of 12 animals each (Figure 1). A group of OVX rats was left untreated (untreated OVX). Both the untreated OVX and the sham OVX groups were used as controls. The different treatments [genistein aglycone (1 and 10 $mg \cdot kg^{-1}$), 17-α-ethinyl oestradiol (0.003 and 0.03 $mg \cdot kg^{-1}$), raloxifene hydrochloride (0.05 and 0.5 $mg \cdot kg^{-1}$)] were administered subcutaneously daily for 12 weeks. The animals were killed by overdose of anaesthetic.

The wound was produced on the back of all animals 14 or 7 days before killing, as previously described (Galeano *et al*., 2008). Briefly, after deep anaesthesia with ether, the rats became unconscious, hair on the back was shaved and skin was washed with povidone-iodine solution and wiped with sterile water. Two full-thickness longitudinal incisions (4 cm) were made on the dorsum of the rat, and the wound edges were closed with 4-0 silk surgical suture placed at 1 cm inter-

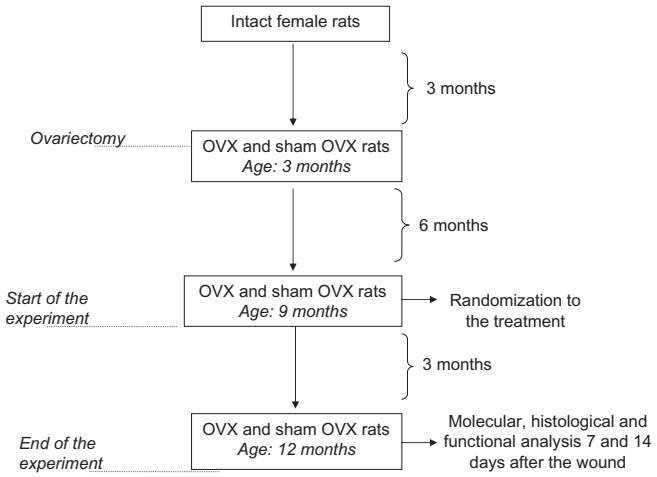

Figure 1 Scheme of the experimental protocol.

vals. The skin was removed by using a scalpel to cut the shape of an ellipse around the lesion. At the end of the experiment, the wounds were removed, tested for breaking strength (only day 14) and subdivided into three segments. The central strip was used for histology (only day 14), and the other segments for molecular analysis.

Unwounded skin samples, used for comparisons, were obtained as biopsies from either untreated sham and OVX rats on the days of the wounding procedures.

Determination of TGF-β1 and tissue transglutaminase 2 (TG2) by Western blot analysis

Briefly, skin samples were homogenized in 1 mL lysis buffer (20 mM HEPES, pH 7.6, 1.0 mM dithiothreitol, 1.0 mM EGTA, 1% Triton, 50 mM β-glycerol phosphate, 10% glycerol, 0.5 mM phenyl methylsulphonyl fluoride, aprotinin, leupeptin, pepstatin A (10 $\mu g \cdot mL^{-1}$ each) and 100 mM Na_3VO_4), with an Ultra-Turrax homogenizer (IKA Company, Staufen, Germany). The homogenate was subjected to centrifugation at 15 000× *g* for 15 min. The supernatant was collected and used for protein determination using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA).

Protein sample (30 μg) was denatured in reducing buffer (62 mM Tris/HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.003% bromophenol blue) and separated by electrophoresis on an SDS (12%) polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane using the transfer buffer (39 mM glycine, 48 mM Tris, 20% methanol) at 100 mA for 1 h. Membranes were stained with Ponceau S (0.005% in 1% acetic acid) to confirm equal amounts of protein, and blocked with 5% non-fat dry milk in TBS–0.1% Tween for 1 h at room temperature, washed three times for 10 min each in TBS–0.1% Tween and incubated with a primary antibody for TGF-β1 (Upstate, NY, USA) and TG2 (Cell Signaling, Beverly, MA, USA) in TBS–0.1% Tween overnight at 4°C, diluted 1:500. After being washed three times for 10 min each in TBS–0.1% Tween, the membranes were incubated with a secondary antibody peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pierce, Rockford, IL, USA) for 1 h at room temperature diluted 1:20 000. After washing, the membranes were analysed by the enhanced chemiluminescence system, according to the manufacturer's protocol (Amersham, Little Chalfont, UK). The protein signal was quantified by scanning densitometry using a bio-image analysis system (Bio-Profil Celbio, Milan, Italy). Equal loading of protein was assessed on stripped blots by immunodetection of β-actin with a rabbit monoclonal antibody (Cell Signaling) diluted 1:500 and peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pierce) diluted 1:15 000. All antibodies were purified by protein A and peptide affinity chromatography.

Determination of vascular endothelial growth factor (VEGF) in wounds

Briefly, tissues were homogenized in 1.0 mL of 1× PBS containing Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN, USA). Homogenates were centrifuged to remove debris, and filtered through a 1.2 m pore syringe filter. Analysis was performed with a commercially available human VEGF-specific enzyme-linked immunosorbent assay kit. The amount of VEGF was expressed as pg (mg^{-1} protein).

Breaking strength

The maximum load (breaking strength) tolerated by wounds was measured without knowledge of treatments on coded samples using a calibrated tensometer (Sans, Milan, Italy) as described previously (Galeano *et al*., 2008). The ends of the skin strip were pulled at a constant speed (20 $cm \cdot min^{-1}$), and breaking strength was expressed as the mean maximum level of tensile strength in newton (N) before separation of wounds.

Histological evaluation

The skin samples were fixed in 10% buffered formalin for light microscopic examination. After fixation, perpendicular sections to the anterior–posterior axis of the wound were dehydrated with graded ethanols and embedded in paraffin. Sections (5 μm thick) were mounted on glass slides, rehydrated to distilled water and stained with haematoxylin and eosin or Masson's trichrome. As part of the histological evaluation, all slides were examined by a pathologist without knowledge of the previous treatment, using masked slides under the microscope from ×5 to ×40 magnification. The following parameters were evaluated: epidermal and dermal healing, granulation tissue formation and angiogenesis. The margins of the wound in each of the sections, as well as normal control wounds, were used as comparison for scoring (Table 1). Concerning angiogenesis, only mature vessels that contained erythrocytes were counted. To evaluate well-formed from poorly formed capillary vessels, the following parameters were considered: presence or absence of oedema, congestion, haemorrhage, thrombosis and intravascular or intervascular fibrin formation. The histological score adopted in this study was evaluated according to data regarding wound healing in experimental models (Galeano *et al*., 2008).

Statistical analysis

All data are expressed as means ± SD. Comparisons between different treatments were analysed by one-way ANOVA fol-

Table 1 Criteria to evaluate histological scores of wound healing

Score	*Epidermal and dermal regeneration*	*Granulation tissue thickness*	*Angiogenesis*
1	Little epidermal and dermal organization	Thin granulation layer	Altered angiogenesis (one to two vessels per site) characterized by a high degree of oedema, haemorrhage, occasional congestion and thrombosis
2	Moderate epidermal and dermal organization	Moderate granulation layer	Few newly formed capillary vessels (three to four per site), moderate degree of oedema and haemorrhage, occasional congestion and intravascular fibrin deposition, absence of thrombosis
3	Complete remodelling of epidermis and dermis	Thick granulation layer	Newly formed capillary vessels (five to six per site), moderate degree of perivascular and interstitial oedema and congestion, absence of thrombosis and haemorrhage
4	–	Very thick granulation layer	Newly formed and well-structured capillary vessels (more than seven per site) vertically disposed towards the epithelium and at the wound margins, slight degree of perivascular oedema

lowed by Tukey's multiple comparison test. In all cases, a probability of less than 0.05 was selected as the criterion for statistical significance. Graphs were drawn using GraphPad Prism (version 4.0 for Windows).

Materials

Genistein aglycone was a kind gift from Primus Pharmaceuticals Inc, Scottsdale, AZ, USA; 17-α-ethinyl oestradiol and raloxifene hydrochloride were purchased from Sigma Aldrich (Milan, Italy). All substances were prepared fresh daily and administered in a volume of 100 μL. The vehicle used to solubilize genistein aglycone, raloxifene and oestradiol was DMSO in 0.9% NaCl solution.

Results

Effect of different treatments on TGF-β1 expression

Unwounded skin samples, used for comparisons, obtained as biopsies from either untreated sham and OVX rats immediately before the beginning of the wounding procedure revealed a reduced content of TGF-β1 in the OVX animals (Figure 2). At day 7 after wounding, OVX untreated animals had a significant reduction of TGF-β1 expression compared with sham OVX (Figure 2). At day 14 after wounding, treatment with 17-α-ethinyloestradiol enhanced significantly TGF-β1 expression at both doses (0.003 and 0.03 mg·kg^{-1} s.c.; E1 and E2 respectively). Genistein aglycone administration at both doses (1 and 10 mg·kg^{-1} s.c.; G1 and G2, respectively) showed a significant increase of TGF-β1 expression. There were no changes of expression of TGF-β1 after treatment of animals with the higher dose of raloxifene hydrochloride (0.5 mg·kg^{-1} s.c.; R2). Overall, comparing results for each therapy, at day 14 after wounding, the lower dose of genistein aglycone (1 mg·kg^{-1} s.c.; G1) showed a greater increase in expression of TGF-β1 than all the other treatments ($P < 0.001$) (Figure 2).

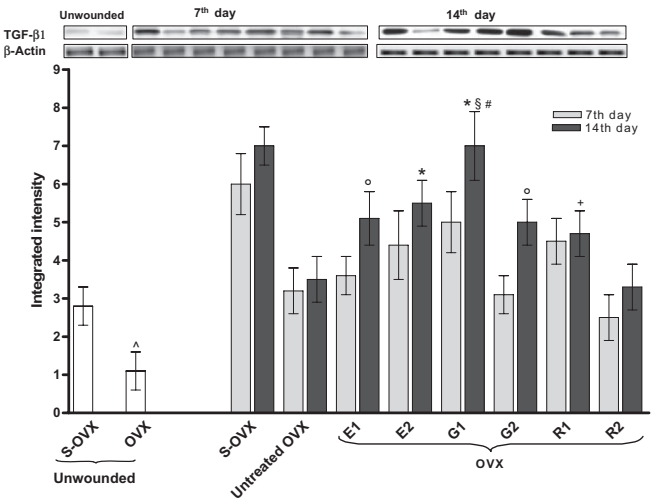

Figure 2 Representative Western blot analysis of TGF-β1 in wounds from OVX rats treated daily with 17-α-ethinyloestradiol at 0.003 and 0.03 mg·kg^{-1} s.c. (E1 and E2, respectively); genistein aglycone at 1 and 10 mg·kg^{-1} s.c. (G1 and G2, respectively); raloxifene hydrochloride at 0.05 and 0.5 mg·kg^{-1} s.c. (R1 and R2 respectively). The effects of different treatments were evaluated on the 7th or 14th day after wounding. The upper panel shows representative autoradiography, highlighting TGF-β1 and β-actin (control) expression. The lower panel shows quantitative data and represents the mean ± SD of six animals. $^{*}P < 0.001$ versus untreated OVX; $^{\circ}P < 0.01$ versus untreated OVX; $^{+}P < 0.05$ versus untreated OVX; $^{\S}P < 0.001$ versus E1,G2,R1,R2; $^{\#}P < 0.01$ versus E2; $^{\wedge}P < 0.001$ versus unwounded sham OVX (S-OVX).

Effect of the treatments on TG2 expression

Unwounded skin samples, used for comparisons, obtained as biopsies from either untreated sham and OVX rats immediately before the beginning of the wounding procedure revealed a reduced content of TG2 in the OVX rats (Figure 3). At day 7 after wounding, TG2 expression was unchanged in all groups (Figure 3). At day 14 after wounding, OVX untreated animals showed a significant reduction of TG2 expression compared with sham OVX. Both doses of genistein aglycone (1 and 10 mg·kg^{-1} s.c.; G1 and G2, respectively) produced a significant, marked increase in TG2 (Figure 3). Raloxifene hydrochloride administration at the dose of 0.05 mg (R1) also significantly increased ($P < 0.01$) TG2 expression compared with untreated OVX rats.

Effect of the treatments on VEGF production in wounds

Unwounded skin samples, used for comparisons, obtained as biopsies from either untreated sham and OVX rats immedi-

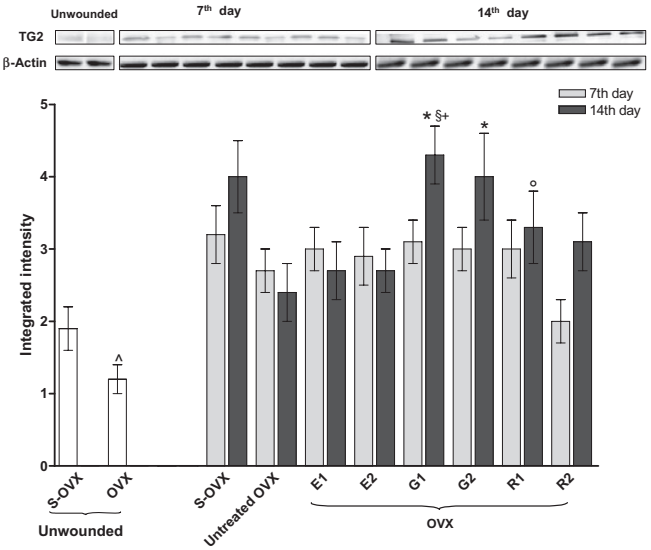

Figure 3 Representative Western blot analysis of TG2 in wounds from OVX rats treated daily with 17-α-ethinyloestradiol at 0.003 and 0.03 mg·kg^{-1} s.c. (E1 and E2, respectively); genistein aglycone at 1 and 10 mg·kg^{-1} s.c. (G1 and G2, respectively); raloxifene hydrochloride at 0.05 and 0.5 mg·kg^{-1} s.c. (R1 and R2 respectively). The effects of different treatments were evaluated on the 7th or 14th day after wounding. The upper panel shows representative autoradiography, highlighting TG2 and β-actin (control) expression. The lower panel shows quantitative data and represents the mean ± SD of six animals. *P < 0.001 versus untreated OVX; °P < 0.05 versus untreated OVX; §P < 0.001 versus E1,E2,R2; +P < 0.01 versus R1; ^P < 0.001 versus unwounded sham OVX (S-OVX).

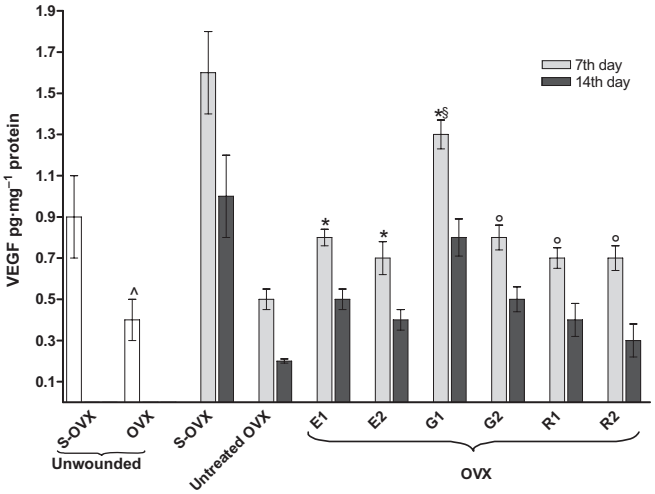

Figure 4 VEGF levels in wounds from OVX rats treated daily with 17-α-ethinyloestradiol at 0.003 and 0.03 mg·kg^{-1} s.c. (E1 and E2, respectively); genistein aglycone at 1 and 10 mg·kg^{-1} s.c. (G1 and G2, respectively); raloxifene hydrochloride at 0.05 and 0.5 mg·kg^{-1} s.c. (R1 and R2 respectively).The effects of different treatments were evaluated on the 7th or 14th day after wounding. The panel shows quantitative data and represents the mean ± SD of six animals. *P < 0.001 versus untreated OVX; °P < 0.01 versus untreated OVX; §P < 0.001 versus all other treatments; ^P < 0.001 versus unwounded sham OVX (S-OVX).

ately before the beginning of wounding procedure revealed a reduced content of VEGF in the OVX animals (Figure 4). At day 7, wounds obtained from OVX-untreated rats showed a marked reduction in VEGF content when compared to normal skin and wounds from sham OVX rats. At day 14, this angiogenic factor was still slightly present in wounds from untreated OVX (Figure 4). Genistein aglycone at the lower dose (1 mg·kg^{-1} s.c.; G1) markedly enhanced the VEGF content of wounds in OVX rats at day 7 (P < 0.001 vs. untreated OVX) and day 14 compared to all the other treatments (P < 0.001) (Figure 4).

Effect of the treatments on skin breaking strength

Untreated OVX animals had significantly reduced skin breaking strength compared with sham OVX rats, at 14 days. Treatment with both doses of 17-α-ethinyloestradiol (0.003 and 0.03 mg·kg^{-1} s.c.; E1 and E2, respectively) significantly reduced skin breaking strength compared with other treatments, while raloxifene hydrochloride administration at the dose of 0.05 mg (R1) significantly increased the breaking strength. Both doses of genistein aglycone (1 and 10 mg·kg^{-1} s.c.; G1 and G2, respectively) produced a greater increase (P < 0.001) in wound resistance than all the other treatments (Figure 5A).

Effects of the treatments on histological parameters

Figure 5B shows the histological score of wounds according to the criteria of Table 1. In sham OVX animals, dermis remodelling and wound closure process were complete after 14 days (Figure 6A). Histological evaluation of incisional wounds in OVX-untreated rats at day 14 disclosed incomplete re-epithelialization, rested on a poorly formed and immature granulation tissue (Figure 6B). Treatment with both doses of 17-α-ethinyloestradiol (0.003 and 0.03 mg·kg^{-1} s.c.) resulted in improved wound healing in OVX rats (Figure 6C,D). Specifically, Figure 6C clearly represents a histological picture characterized by adequate re-epithelialization and new collagen deposition. By contrary, raloxifene hydrochloride administration (0.05 mg·kg^{-1}) allowed an adequate wound closure process (Figure 6E), while the higher dose of raloxifene hydrochloride (0.5 mg·kg^{-1} s.c.) induced an incomplete wound healing with persistence of a partially organized granulation tissue and inflammatory cells, 14 days following surgery (Figure 6F). Indeed, the histological score of wound healing indicated that both doses of genistein aglycone (1 and 10 mg·kg^{-1} s.c.) qualitatively and quantitatively improved wound healing at day 14 (Figure 5B). Specifically, comparing results for each therapy, at day 14 after wounding, genistein aglycone at the lower dose showed better results. As shown in Figure 6G,H, the histological picture is characterized by a complete re-epithelialization, a well-formed granulation tissue and a good presence of collagen in skin of OVX rats treated with genistein aglycone.

Masson's trichrome stain for collagen showed a thin and altered collagen layer in OVX-untreated animals due to ageing and oestrogen loss compared to sham OVX (Figure 7A,B). All the treatments, at the low-dose regimens, significantly augmented the thickness of collagen tissue (Figure 7C–F). Genistein aglycone also restored the altered architecture in collagen layers, especially at 1 mg·kg^{-1}.

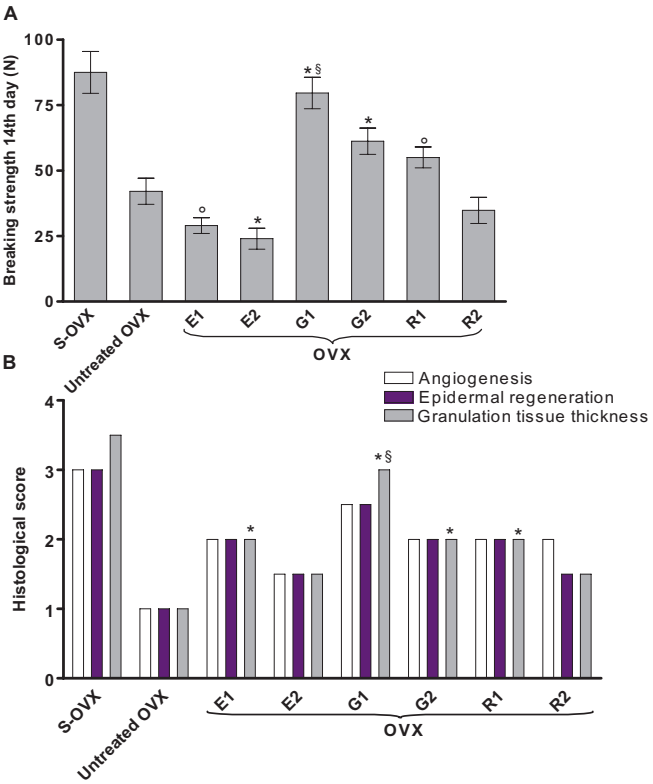

Figure 5 Effects of 17-α-ethinyloestradiol at 0.003 and 0.03 mg·kg^{-1} s.c. (E1 and E2, respectively); genistein aglycone at 1 and 10 mg·kg^{-1} s.c. (G1 and G2, respectively); raloxifene hydrochloride at 0.05 and 0.5 mg·kg^{-1} s.c. (R1 and R2, respectively) on skin breaking strength (A) and histological score (B) in OVX rats. Data are shown as the mean ± SD of six animals. *$P < 0.001$ versus untreated OVX; °$P < 0.01$ versus untreated OVX; §$P < 0.001$ versus all other treatments.

Discussion

Under conditions of reduced systemic oestrogen production, such as menopause or ovariectomy, wound healing is delayed, local inflammation increased and collagen deposition decreased. In humans, these defects may be reversed through HRT or the topical administration of 17β-oestradiol (Brincat *et al*., 1985; Varila *et al*., 1995; Ashcroft *et al*., 1997a; 1999); however, despite such and other beneficial effects of oestrogens, HRT cannot be recommended to all menopausal women. Moreover, topical oestrogen treatment for skin diseases will need to be administered by a skilled dermatologist given that concentration and application areas must be specifically observed in order to avoid systemic side effects and respect the physiology of the skin.

In light of this background, SERMs and other alternative therapeutic approaches have been considered to prevent or treat effects related to oestrogen loss. Specifically, much emphasis has been placed on some phyto-oestrogens, particulary isoflavones, which have tissue-specific oestrogen actions that can separate the positive biological effects from the undesirable effects (Accorsi-Neto *et al*., 2009; Moraes *et al*., 2009). So far, as the majority of observations on skin healing properties have been made following acute therapies, started immediately after wounding, in the present experiment we chose to investigate the long-term effect of several treatments in an incisional wound healing model.

Here, we demonstrated for the first time that chronic treatment with the isoflavone, genistein aglycones, was able to counteract delayed wound healing, improving extracellular matrix remodelling and turn-over in OVX rats. Moreover, genistein aglycone was shown to be more effective than oestradiol or raloxifene on all skin parameters tested at days 7 and 14 after wounding.

In this context, molecular data regarding TGF-β1 and TG2 expression in wounds from OVX rats utilized in our experiments are encouraging. Indeed, TGF-β1 can present contradictory effects on skin, but it is well known that this mediator, released by cells that are localized at sites of tissue repair, such as platelets, activated macrophages and possibly fibroblasts, exerts beneficial effects in each phase of wound healing (Grande, 1997; Klass *et al*., 2009). Thus, TGF-β1 in the inflammatory phase is necessary for proper chemoattraction of monocytes; in the proliferative phase, it stimulates extracellular matrix production and affects angiogenesis and epithelialization, and, finally, induces contraction and myofibroblast formation in skin wounds. Recently, in aged animals, TGF-β1 increased the rate of healing and the breaking strength of the repaired tissue, and it also enhanced angiogenesis and consequent blood flow to dermal wounds, partly by stimulating the local release of other growth factors (Adams *et al*., 2008). An *in vitro* study (Merlo *et al*., 2009) suggested that activation of the ER-β was sufficient to obtain a stimulatory effect on wound repair through a mechanism that did not involve excessive production of TGF-β1. However, our data show an increase in TGF-β1 following genistein aglycone administration only when compared to untreated OVX, not to sham OVX rats. As stimulation of ER-β results in enhanced keratinocyte proliferation and migration, the augmented TGF-β1 levels might originate from a prolonged stimulation of these cells as our experiment lasted for a relatively long period. In this context, it would be of interest to verify this hypothesis in an excisional model of wound healing. As a matter of fact, an excisional wound healing model would allow us to better understand whether genistein aglycone, through TGF-β1 and/or via other non-ER-mediated pathways could also be able to differentiate fibroblast into myofibroblasts, and promoting an adequate wound contraction in the maturational phase. Of course, additional experiments in order to further clarify this mechanism of action will be part of our future studies.

TG2 has both direct and indirect crucial effects on the extracellular matrix, either through direct protein cross-linking leading to matrix stabilization or indirectly via the activation of TGF-β1, leading, in turn, to matrix deposition (Griffin *et al*., 2002). TG2 can also act as an independent cell adhesion protein when bound to fibronectin, preventing cell death by anoikis (Verderio *et al*., 2003). The end result is an appropriate wound healing and maintenance of tissue integrity, as demonstrated by histological score and enhanced breaking strength in genistein-treated OVX rats. Surprisingly, genistein aglycone restored wounded skin as well or better than other commonly accepted treatments for improving skin trophism. Stimulation of TGF-β1 and TG2 following genistein aglycone administration might also explain the

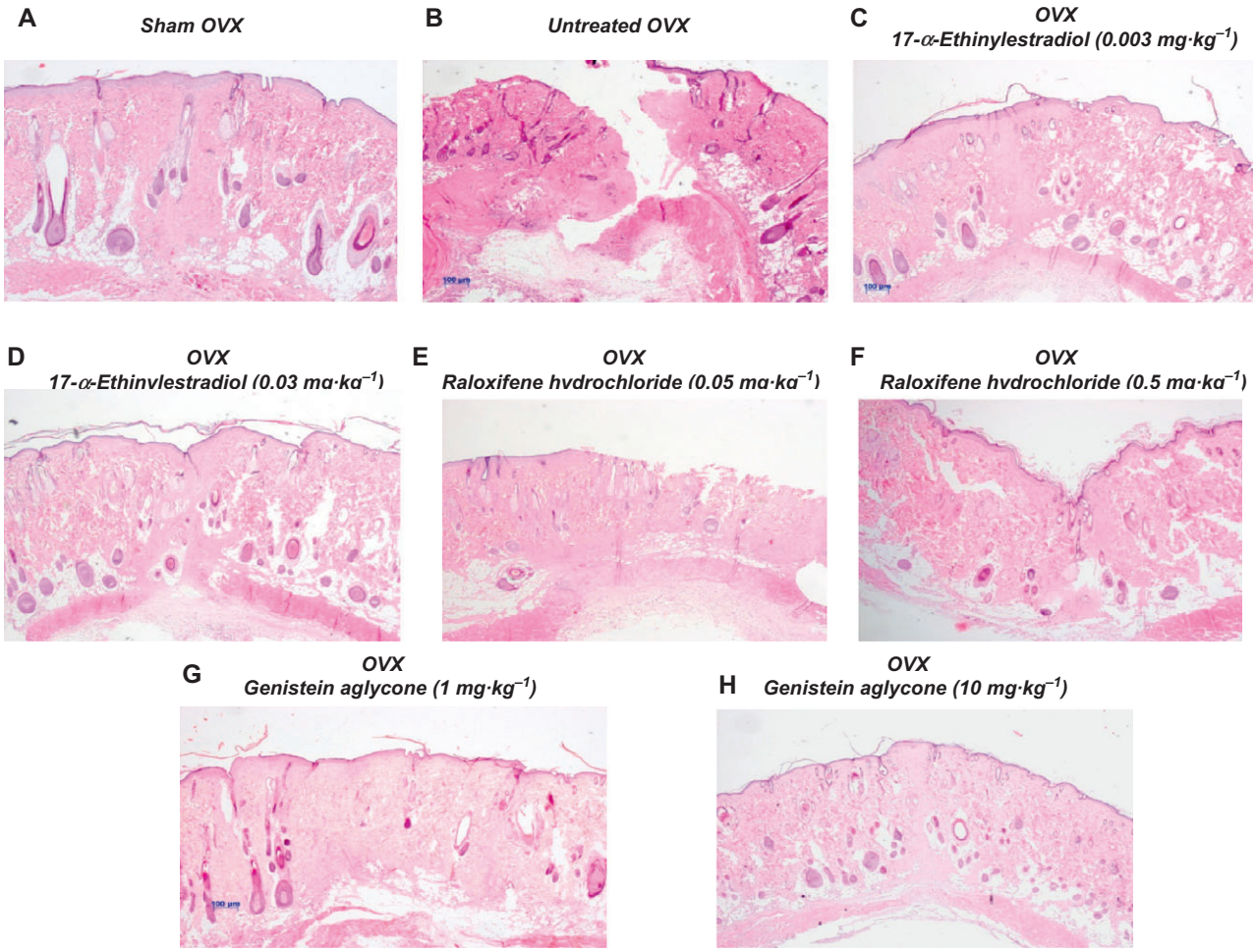

Figure 6 (A–H) Light microscopy of incisional wounds in OVX rats: effects of 17-α-ethinyloestradiol (0.003 and 0.03 mg·kg^{-1} s.c.), raloxifene hydrochloride (0.05 and 0.5 mg·kg^{-1} s.c.) and genistein aglycone (1 and 10 mg·kg^{-1} s.c.); (haematoxylin and eosin, ×5 original magnification).

increased content of VEGF in wounds. This finding is very intriguing: in fact, VEGF has a pivotal role in the initiation of angiogenesis based on its ability to induce the expression of proteases that digest components of the extracellular matrix that impede angiogenesis, to promote endothelial cell proliferation and to prevent their apoptosis (Bao *et al*., 2009). Indeed, during normal healing, VEGF is commonly produced by neutrophils, macrophages and fibroblasts, whereas under conditions like oestrogen loss and/or ageing, deregulation of VEGF may impair healing by reducing the inflammatory response, as well as the number of fibroblasts in skin. In this context, our data clearly agree with previous reports showing that oestrogens facilitate wound healing and increase VEGF in several tissues (Mowa *et al*., 2008; Stevenson *et al*., 2008).

In this experimental model, the observed molecular effects achieved by chronic treatment with genistein aglycone correlated very well with each histological parameter considered, including epidermal regeneration; thickness of the granulation tissue; formation of fibroblasts; and new, well-structured capillary vessels. Indeed, the lowest genistein dose was more effective in accelerating wound healing than raloxifene hydrochloride or oestradiol 14 days following wounding. This latter finding confirms further that cell migration, inflammation, provisional matrix synthesis, collagen deposition, angiogenesis and re-epithelialization are crucial in the process of skin repair improved by genistein administration. In addition, whereas collagen tissue was found extremely reduced by oestrogen loss, ageing and wounding in untreated OVX animals, all treatments restored the thickness of the collagen layer following wounding, but genistein aglycone was the only treatment that clearly also re-established its altered architecture.

Finally, genistein aglycone was also the most beneficial treatment in terms of skin functionality. It is often reported that in menopausal women, changes in skin collagen lead to reduced elasticity and skin strength. In fact, the breaking strength of incisional wounds of rats treated with subcutaneous injection of genistein was higher than that of rats treated with raloxifene hydrochloride or oestradiol. This latter suggests that genistein through its multiple actions can also restore the mechanical properties of damaged skin.

Our experimental data indicated that two doses of genistein aglycone, given continuously by subcutaneous injection in OVX rats, were able to produce positive effects on wound healing in the same range of dosing, as that used in postmenopausal women treated orally with 54 mg per day of

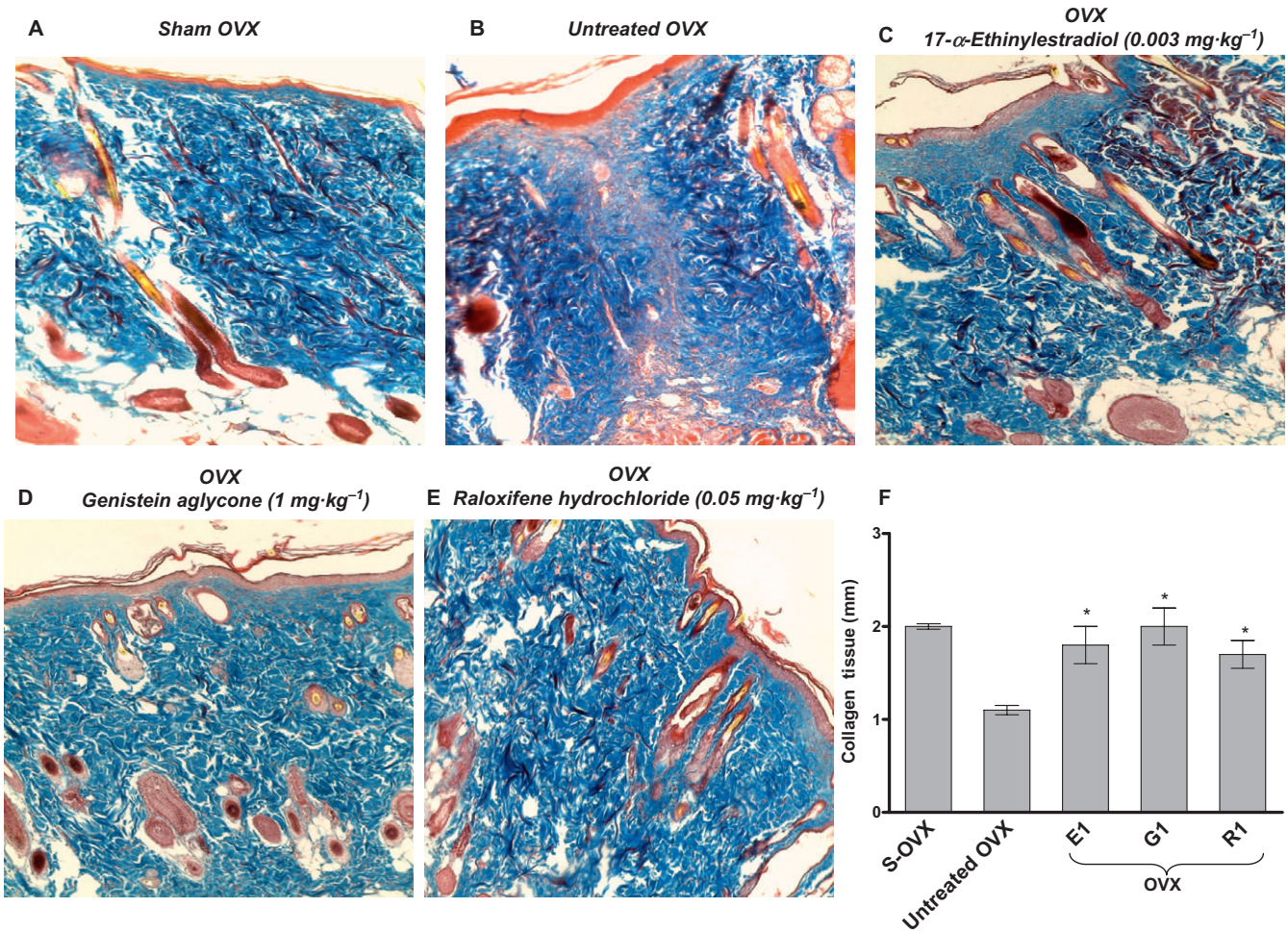

Figure 7 (A–E) Light microscopy of incisional wounds in OVX rats (Masson's trichrome stain, ×10 original magnification) and (F) quantification of collagen tissue. Representative data describing collagen tissue thickness are shown as the mean ± SD of six animals. *$P < 0.001$ versus untreated OVX.

aglycone genistein (Squadrito *et al.*, 2003; Atteritano *et al.*, 2007; Marini *et al.*, 2007;2008a,b; 2009; D'Anna *et al.*, 2009). So far, the hypothesis that the present experimental findings might be related to an acute effect of genistein can be easily ruled out because in previous experiments (data not shown), we did not observe any significant effect of genistein administration in OVX rats immediately treated for 14 days after wounding.

It is very likely that these positive findings following treatment with systemic genistein aglycone could be related to selective activation of the ER-β, particularly in the stratum basalis, stratum spinosum, stratum granulosum and papillary dermis of OVX rat skin (Verdier-Sévrain *et al.*, 2006).

In fact, unlike 17β-oestradiol, which displays relatively equal binding to both ER subtypes, and raloxifene, which binds with greater affinity to the ER-α, genistein aglycone binds preferentially to ER-β over ER-α in an oestrogen-deprived environment, and this selectivity was 7- to 48-fold, depending on the assay system employed (Kuiper *et al.*, 1997; 1998; Barkhem *et al.*, 1998; Hsieh *et al.*, 2006). Our experimental data also show that the concentration of genistein aglycone must be properly titrated, as some reports suggest that an insufficient stimulation of ER-β may occur, leading to few or even absent beneficial effects, at low doses (Makela *et al.*, 1999; Setchell, 2001; Kostelac *et al.*, 2003; Altavilla *et al.*, 2004; McCarty, 2006). Indeed, at higher concentrations, genistein aglycone binds to ER-α, and, as previously observed in other target tissues (i.e. bone, endothelium), it could minimize the overall positive effects on wound healing obtained through ER-β binding. Additionally, it is necessary to underline that the observed effects of genistein on skin wound healing could be related to a range of non-genomic actions on skin cells, as well demonstrated in other target tissues (Atmaca *et al.*, 2008).

Overall, our results strongly suggest that genistein aglycone might be a new potential therapy for the management of skin wound healing in post-menopausal women. However, a comparison between systemic and topical administration of genistein aglycone will be needed in addition to further experiments in order to clarify if genistein aglycone may also represent a novel therapeutic approach to other dermatological disorders in humans.

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Conflicts of interest

All the authors have none to declare.

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