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RESEARCH PAPER

Genistein aglycone, a soy-derived isoflavone, improves skin changes induced by ovariectomy in rats

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BACKGROUND AND PURPOSE

Ovariectomy accelerates age-related skin changes as adequate oestrogen levels are required to control structural integrity and functional capacity of skin. Genistein, a soy-derived isoflavone, has been tested in anti-ageing cosmetic preparations with interesting results on skin elasticity, photoaging and skin cancer prevention. We investigated the effects of genistein aglycone and compared them with systemic raloxifene hydrochloride and 17-α-ethinyloestradiol on skin changes in aged, ovariectomized (OVX) rats.

EXPERIMENTAL APPROACH

Six months after ovariectomy, rats were randomly allocated to different groups 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-ethinyloestradiol (0.003 and 0.03 mg·kg^{-1} s.c.) for 12 weeks. Controls were untreated OVX and sham OVX rats. At the end of the treatment period, a skin biopsy was carried out and skin samples were assessed for molecular, histological and functional changes.

KEY RESULTS

Skin samples of untreated OVX rats showed a decrease in TGF-β1, VEGF, MMP-2, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 compared with sham OVX rats. All the treatments significantly restored this depressed molecular profile revealed in OVX rats. Genistein aglycone, 1 mg·kg^{-1}, also significantly increased the thickness of collagen and breaking strength of skin in the OVX rats.

CONCLUSIONS AND IMPLICATIONS

Relatively long-term, systemic treatment with genistein aglycone shows comparable efficacy to oestrogen in reversing some molecular, histological and functional changes of the skin associated with ovariectomy in aged rats. This suggests that genistein aglycone might be an effective alternative therapy for the management of age-related skin changes in postmenopausal women.

Abbreviations

ER, oestrogen receptor; HRT, hormone replacement therapy; OVX, ovariectomized; SERM, selective oestrogen receptor modulator; ECM, extracellular matrix; TIMP, tissue inhibitor of metalloproteinase

Introduction

Age-related skin changes include a decrease in skin thickness due to atrophy of the epidermis, dermis and subcutaneous fat, dryness, wrinkling and an increased incidence of proliferative lesions. Specifically, in the epidermis, ageing causes a reduction in epidermal thickness, flattening of the dermal papilla and a decrease in melanocyte and Langerhans cell density. Furthermore, within the dermis, it has been also shown that ageing decreases fibroblast activity leading to a reduced collagen and hyaluronic acid content, more fragmented elastin fibres and decreased vascularity. Unfortunately, menopause accelerates these skin changes as adequate oestrogen levels are required to control structural integrity and functional capacity of skin (Verdier-Sévrain *et al.*, 2006). Experimental data show that skin elasticity decreases significantly 3–13 weeks after ovariectomy, accompanied by a significant increase in elastase activity in the skin (Tsukahara *et al.*, 2004). Like collagen, elastin is a fibre-forming functional protein. Elastin fibres are closely linked and interwoven with the collagen fibrils so that they can recoil after transient stretching and are prevented from overstretching. Young women who underwent premature menopause were observed to have accelerated degenerative changes in dermal elastic fibres (Bolognia *et al.*, 1989). During the ageing process, there is also a decrease in the production of glycosaminoglycans in the connective tissue, and a higher intracellular concentration of protocollagen lysyl-hydroxyproline transferase, which is the enzyme responsible for collagen breakdown (Punnonen *et al.*, 1987). The postmenopausal skin state can be, at least partially, restored by hormone replacement therapy (HRT) or local oestrogen treatment (Piérard-Franchimont *et al.*, 1995; Sator *et al.*, 2001; Shah and Maibach, 2001; Wolff *et al.*, 2005). However, with regard to long-term use of oestrogen supplementation as a specific therapeutic approach for skin changes in menopausal women, perceptions of oestrogen side effects and risk profiles in some women limit the possibility of this use in wider populations. In the light of this, alternative therapeutic approaches have recently been considered that specifically manage the skin changes typically associated with the menopause. In the last decades, selective oestrogen receptor modulators (SERMs) such as raloxifene 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) interactions (Verdier-Sévrain, 2007); however, to date, there is very limited data on the effect of SERMs on the skin.

Asians, who consume high levels of isoflavones in their diet, appear to show different skin damage compared with their Caucasian counterparts (Goh, 1990). A number of studies have included soy isoflavones alone or in combination with other agents and showed positive effects on skin appearance and composition (Skovgaard *et al.*, 2006; Draelos et al., 2007; Izumi *et al.*, 2007; Accorsi-Neto *et al.*, 2009).

Genistein aglycone (hereinafter genistein), an isoflavone found in low concentrations in soybeans and in increased amounts in certain fermented soy foods, has been recently considered as an ideal natural SERM and might play a preventive role in age-related skin changes accelerated by the menopause without the harmful oestrogenic side effects on reproductive tissues (Cassidy, 2003). Genistein showed consistent efficacy in managing conditions of oestrogen deprivation, and its cosmetic creams were actually used to improve skin dryness and wrinkles (Rona *et al.*, 2004). The efficacy and safety of genistein in a low oestrogen environment have already been demonstrated in previous experimental and clinical studies (Squadrito *et al.*, 2003; Atteritano *et al.*, 2007; Marini *et al.*, 2007, 2008a,b, 2010a, 2010b; Bitto *et al.*, 2008, 2009a,b; D'Anna *et al.*, 2009) and this promising therapeutic profile may be a direct consequence of greater genistein affinity for ER-β than ER-α (Kuiper *et al.*, 1998; Harris *et al.*, 2005). In fact, ER-β is more widely distributed within the skin and its structures (Haczynski *et al.*, 2002; Thornton *et al.*, 2003) and, at physiological concentrations, oestrogen should target the selective receptors, ER-α and ER-β, co-expressed in human skin fibroblasts (Haczynski *et al.*, 2002). It is very likely that ER-β-selective ligands such as genistein could play a regulatory role in restoring ovariectomy-impaired skin while achieving an adequate balance between this and the extracellular matrix (ECM) of the skin.

The present study originates from preliminary experiments in ovariectomized (OVX) rats aimed at investigating the effects of genistein, 17-α-ethinyloestradiol and raloxifene hydrochloride on bone loss induced by oestrogen deprivation (Bitto *et al.*, 2008). In these same animals a skin biopsy was carried out after 12 weeks of treatment and skin specimens were collected to evaluate molecular, histological and functional measurements in order to understand the effects of the above-mentioned compounds on skin changes related to oestrogenic deprivation post-ovariectomy.

Methods

Animals and treatments

All animal care and experimental procedures were carried out in accordance 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) and 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, 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, animals were divided into eight groups of 12 animals each. A group of OVX and sham OVX rats were left untreated (untreated OVX). Both the untreated OVX and sham OVX groups were used as controls. The different treatments, genistein (G; 1 and 10 $mg \cdot kg^{-1}$), 17-α-ethinyloestradiol (E; 0.003 and 0.03 $mg \cdot kg^{-1}$), raloxifene hydrochloride (R; 0.05 and 0.5 $mg \cdot kg^{-1}$) were given s.c. daily, for 12 weeks (Figure 1). Indeed, as no significant difference was revealed in samples

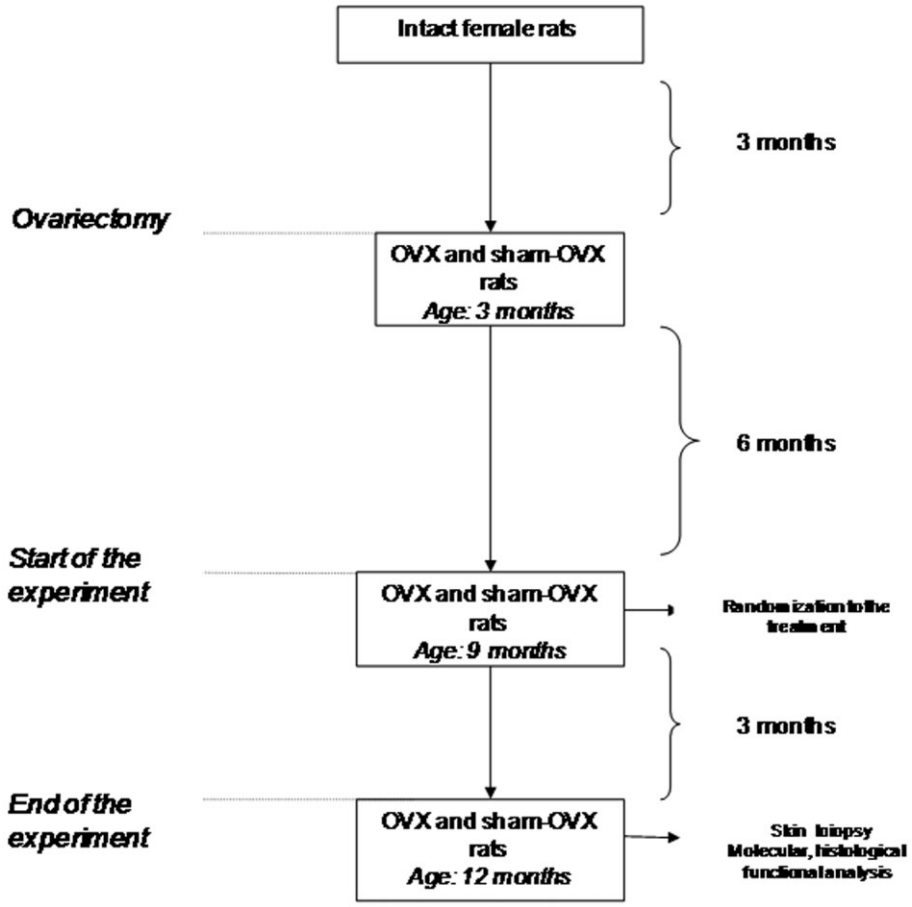

Figure 1
Representative flow chart of experimental model.

obtained from rats treated with two different doses for each drug, in the present paper only the following experimental groups were shown: genistein (1 mg·kg^{-1} s.c.); raloxifene hydrochloride (0.05 mg·kg^{-1} s.c.); or 17-α-ethinyloestradiol (0.003 mg·kg^{-1} s.c.).

After the animals had been deeply anaesthetized with ether, hair on the back was shaved, skin biopsy was performed at the end of the treatment and samples obtained were stored for further analysis. In more detail, the skin was removed by using a scalpel to cut the shape of an ellipse on the back of animal and subdivided into three segments. The central strip was used for histology, and the other segments for molecular analysis and breaking strength.

Genistein dose calculation

The original doses of genistein used in the parent study (1 and 10 mg kg^{-1}) (Bitto *et al.*, 2008) were used to bracket a previous dose (5 mg kg^{-1}) delivered s.c., which also showed bone building effects (Fanti *et al.*, 1998). There are no formal studies that demonstrate an oral equivalence to this dose range for genistein given s.c., but Hertrampf *et al.* (2009) have found similar effects for bone building comparing 10 mg kg^{-1} delivered s.c. with 14 mg kg^{-1} by oral gavage.

Histological and immunohistochemical evaluation

Skin samples were fixed in 10% buffered formalin for light microscopic examination. After fixation, skin sections were dehydrated with graded ethanols and embedded in paraffin. Skin sections of 5 μm thickness were mounted on glass slides, rehydrated with distilled water and stained with Masson's trichrome. Masson's trichrome stains collagen fibres in the form of dense blue deposits; these were submitted for image analysis using Leica Quin (Leica Imaging System, Ltd., Cambridge, UK), which consisted of a personal computer connected to a microscope. The image analyser was first calibrated to convert the measurement unit produced by the image analyser program (pixels) into actual μm units. Five fields from each section were selected and the % of the area with dense blue deposits was measured by the image analyser in relation to a standard measuring frame, which was 7286.8 μm^2, using magnification ×400. The area % obtained from the image analyser was subjected to statistical analysis. For immunohistochemistry, paraffin-embedded tissues were sectioned (5 μm), and antigen retrieval was performed using 0.05 M sodium citrate buffer. Tissues were treated with primary antibody against MMP-2 or MMP-9

(Thermo Scientific, West Sussex, UK). Secondary antibody was provided by Innovex (Richmond, CA, USA), and the location of the reaction was visualized with diaminobenzidine tetra-hydrochloride (Sigma, St. Louis, MO, USA). Slides were counterstained with neutral red and mounted with cover slips. As part of the histological evaluation, all slides were examined by a pathologist who had no knowledge of the previous treatment, using masked slides ×5 magnification with a Zeiss microscope.

Determination of TGF-β1, VEGF, MMP-2, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 by Western blot analysis

Briefly, skin samples were homogenized in 1 mL lysis buffer (20 mM HEPES, pH 7.6, 1.0 mM DTT,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. 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 samples (30 µg) were 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 Tris-buffered saline (TBS)-0.1% Tween for 1 h at room temperature, washed and incubated with a primary antibody for TGF-β1 (Cell Signaling, Beverly, MA, USA), MMP-2 and MMP-9 (Thermo Scientific), TIMP-1 and TIMP-2 (Abcam, Cambridge, UK) and VEGF (Cell Signaling) in TBS-0.1% Tween overnight at 4°C. After being washed the membranes were incubated with a peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA) for 1 h at room temperature. After being washed, the membranes were analysed by using an 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) and peroxidase-conjugated secondary antibody (Pierce). All antibodies were purified by protein A and peptide affinity chromatography.

Breaking strength

The maximum load (breaking strength) tolerated by skin samples was measured by a person unaware of the 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).

Statistical analysis

All data are expressed as the mean ± SD and were normally distributed with equal variance between the groups. Comparisons between different treatments were analysed by one-way ANOVA followed by Tukey's multiple comparison test. In all cases, a *P*-value of less than 0.05 was selected as the criterion for statistical significance. Graphs were drawn using GraphPad Prism (version 4.0 for Windows, GraphPad, San Diego, USA).

Drugs

Genistein was a kind gift from Primus Pharmaceuticals Inc. (Scottsdale, AZ, USA); 17-α-ethinyloestradiol and raloxifene hydrochloride were purchased from Sigma Aldrich (Milan, Italy).

All substances were prepared fresh daily and administered s.c. at a volume of 100 µL. The vehicle used to dissolve all substances was 33% DMSO in 0.9% NaCl.

Results

Effects of the treatments on histological parameters

Ovariectomy markedly affected skin histology: in fact, collagen thickness was significantly reduced in untreated OVX compared with sham OVX untreated rats ($P < 0.001$) as revealed by Masson's trichrome staining (Figure 2A,B and F). Treatment with 17-α-ethinyloestradiol ($0.003\ mg \cdot kg^{-1}$ s.c.), genistein ($1\ mg \cdot kg^{-1}$ s.c.) or raloxifene hydrochloride ($0.05\ mg \cdot kg^{-1}$ s.c.) significantly increased collagen thickness (both 17-α-ethinyloestradiol and genistein $P < 0.001$ vs. untreated OVX; raloxifene $P < 0.01$ vs. untreated OVX Figure 2C, 2D, 2E and 2F); genistein algycone also restored the altered skin architecture at the dose tested.

Effect of different treatments on TGF-β1 expression

Skin samples from untreated OVX rats showed a reduced content of TGF-β1 compared with sham OVX ($P < 0.001$; Figure 3A). All the different treatments (17-α-ethinyloestradiol, $0.003\ mg \cdot kg^{-1}$ s.c.; genistein, $1\ mg \cdot kg^{-1}$ s.c.; raloxifene hydrochloride, $0.05\ mg \cdot kg^{-1}$ s.c.) significantly enhanced TGF-β1 expression compared with untreated OVX group (Figure 3A; raloxifene $P < 0.001$ vs. untreated OVX; 17-α-ethinyloestradiol $P < 0.01$ vs. untreated OVX; genistein $P < 0.05$ vs. untreated OVX).

Effect of different treatments on VEGF production

Oestrogen loss following ovariectomy also induced a marked reduction in VEGF in untreated OVX compared with sham OVX rats as shown in Figure 3B ($P < 0.001$ vs. sham OVX); after 3 months of treatment all the compounds administered in the present experiment (17-α-ethinyloestradiol, $0.003\ mg \cdot kg^{-1}$ s.c.; genistein, $1\ mg \cdot kg^{-1}$ s.c.; raloxifene hydrochloride, $0.05\ mg \cdot kg^{-1}$ s.c.) significantly improved VEGF expression in skin samples compared with the untreated OVX

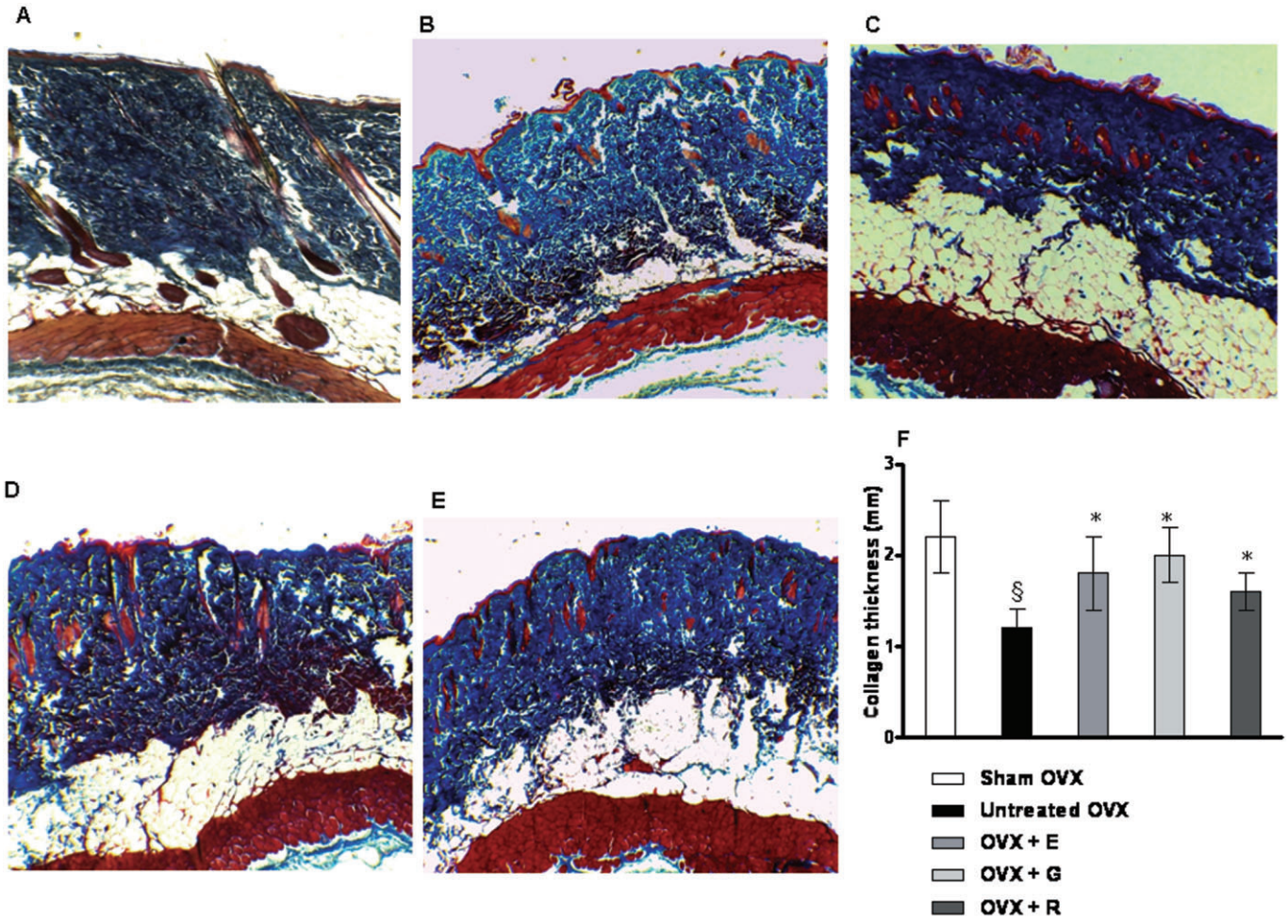

Figure 2

Light microscopy of Masson's tricrome staining in skin biopsies from sham OVX (A), untreated OVX (B), OVX + 17-α-ethinyloestradiol 0.003 mg·kg^{-1} s.c. (C), OVX + genistein 1 mg·kg^{-1} s.c. (D) and raloxifene hydrochloride 0.05 mg·kg^{-1} s.c. (E). Original magnification ×5. (F) Histogram represents the thickness (mm) of the collagen layer; $^{§}P < 0.001$ vs. sham OVX; $^{*}P < 0.001$ vs. untreated OVX.

group (Figure 3B, genistein $P < 0.001$ vs. untreated OVX; 17-α-ethinyloestradiol $P < 0.01$ vs. untreated OVX; raloxifene $P < 0.05$ vs. untreated OVX).

Effect of different treatments on MMP-2, MMP-9, TIMP-1 and TIMP-2 protein expression

Oestrogen loss is associated with an altered balance of ECM proteins as confirmed by Western blot analysis (Figure 4) and immunostaining for MMP-2 (Figure 5) and MMP-9 (Figure 6) in skin biopsies obtained from sham OVX and untreated OVX rats. The imbalance in ECM proteins is not only dependent on MMPs but it is also related to specific inhibitors (TIMPs) of MMPs preventing an uncontrolled ECM degradation. Oestrogen loss following ovariectomy induced a marked reduction in MMP-2, MMP-9, TIMP-1 and TIMP-2 protein expression in untreated OVX rats compared with sham OVX ($P < 0.001$). However, after 3 months of treatment, all the compounds positively restored MMP-2, MMP-9, TIMP-1 and TIMP-2 protein levels (Figure 4A–D; both 17-α-ethinyloestradiol and genistein $P < 0.001$ vs. untreated OVX; raloxifene $P < 0.01$ vs. untreated OVX). ECM proteins expression was estimated by using the mean values ± SD of integrated intensity obtained from Western blot analysis. All treatments markedly increased MMP-2 and MMP-9 staining, as revealed by immunohistochemical analysis. In particular MMP-2 staining was more evident in the dermal layer, while MMP-9 staining was more marked in the epidermal layer of skin in OVX rats as shown in Figures 5C, D, E and 6C, D, E.

Effect of different treatments on skin breaking strength

Untreated OVX animals had significantly reduced skin breaking strength compared with sham OVX rats ($P < 0.001$). The various treatments (17-α-ethinyloestradiol, 0.003 mg·kg^{-1} s.c.; genistein, 1 mg·kg^{-1} s.c.; raloxifene hydrochloride, 0.05 mg·kg^{-1} s.c.) significantly increased the breaking strength of samples obtained by skin biopsies. Inter-

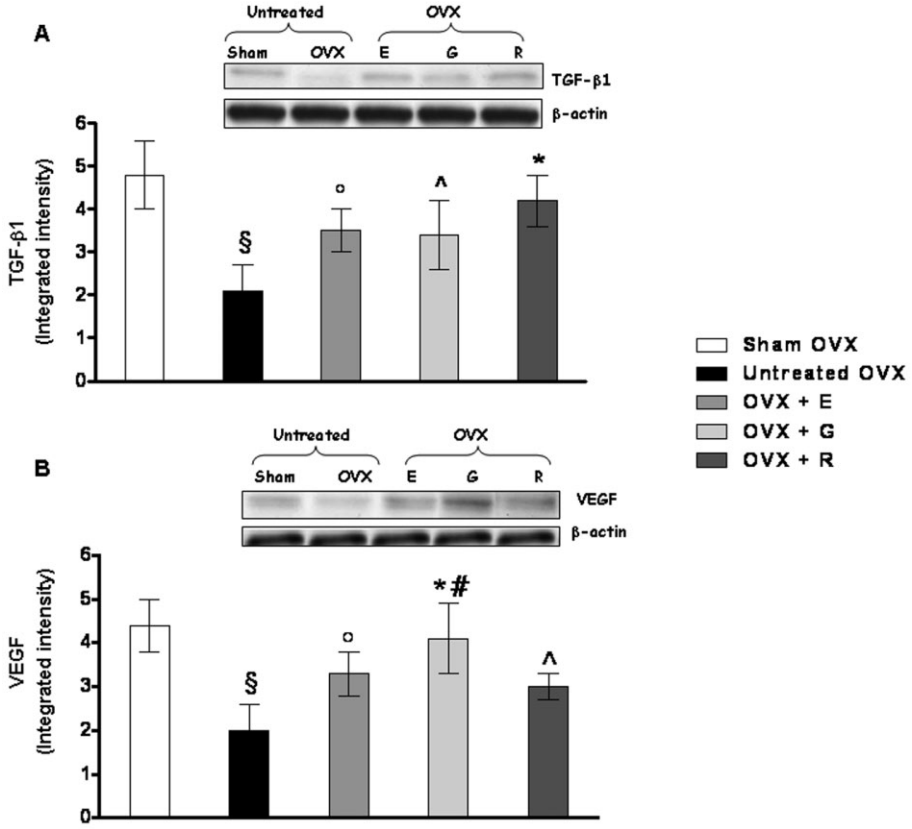

Figure 3

Representative Western blot analysis of both TGF-β1 (A) and VEGF (B) in skin biopsies from ovariectomized rats (OVX) treated, daily, with 17-α-ethinyloestradiol (E; 0.003 mg·kg^{-1} s.c.); genistein (G; 1 mg·kg^{-1} s.c.); raloxifene hydrochloride (R; 0.05 mg·kg^{-1} s.c.). The upper panel shows a representative autoradiography highlighting TGF-β1 and β-actin (control) expression. The columns show quantitative data and represent the mean ± SD of six animals. $^{§}P < 0.001$ vs. sham OVX; $^{*}P < 0.001$ vs. untreated OVX; $^{°}P < 0.01$ vs. untreated OVX; $^{\wedge}P < 0.05$ vs. untreated OVX; $^{\#}P < 0.05$ vs. OVX + raloxifene.

estingly, genistein produced a greater increase ($P < 0.001$) in skin resistance than all the other treatments (Figure 7).

Discussion

It is well known that under conditions of reduced systemic oestrogen production, skin could be structurally and functionally damaged and, as a consequence, skin ageing, impaired wound healing and skin cancer (Calleja-Agius *et al.*, 2007) are increased in menopausal women. So far, these negative conditions may be reversed through HRT but despite such and other beneficial effects of oestrogens, HRT is not suitable for all menopausal women. Moreover, the molecular processes involved and the mechanisms by which oestrogens and related compounds regulate skin function and delay skin ageing have not been fully elucidated. Topical administration of oestrogen is also possible, but it needs to be administered by a skilled dermatologist given that concentration and application areas must be monitored to assess any adverse effects on the physiology of the skin. More emphasis has been placed on some natural compounds, particularly isoflavones, which have tissue-specific oestrogen-like actions without the undesirable side effects (Accorsi-Neto *et al.*, 2009; Moraes *et al.*, 2009). Accordingly, the data presented in this study demonstrate for the first time that a relatively long-term, systemic treatment with genistein shows comparable efficacy to oestrogen in reversing some molecular, histological and functional changes of the skin associated with ovariectomy and ageing in rodents. Further these new data are also presented in comparison with results obtained with a well-known synthetic SERM, raloxifene.

Indeed, as oestrogen levels fall, collagen, elastin and glycosaminoglycans break down, causing sagging, wrinkling and a loss of skin tone and elasticity. Loss of oestrogen also leads to thinning of the skin, reduced blood vessels and increased dryness, fragility and sensitivity to environmental irritants. Both mouse and rat OVX animal models have been utilized to assess the effects of oestradiol on epithelial and on epidermal tissue (Ashcroft *et al.*, 2003; Jelinsky *et al.*, 2008; Campbell *et al.*, 2010). With this in mind, we have utilized an OVX rat model to assess genistein's effect on skin. In our experimental model, following systemic administration of genistein, raloxifene hydrochloride or 17-α-ethinyloestradiol, we observed an increased collagen thickness and, consequently, the architecture of skin was restored compared with untreated OVX

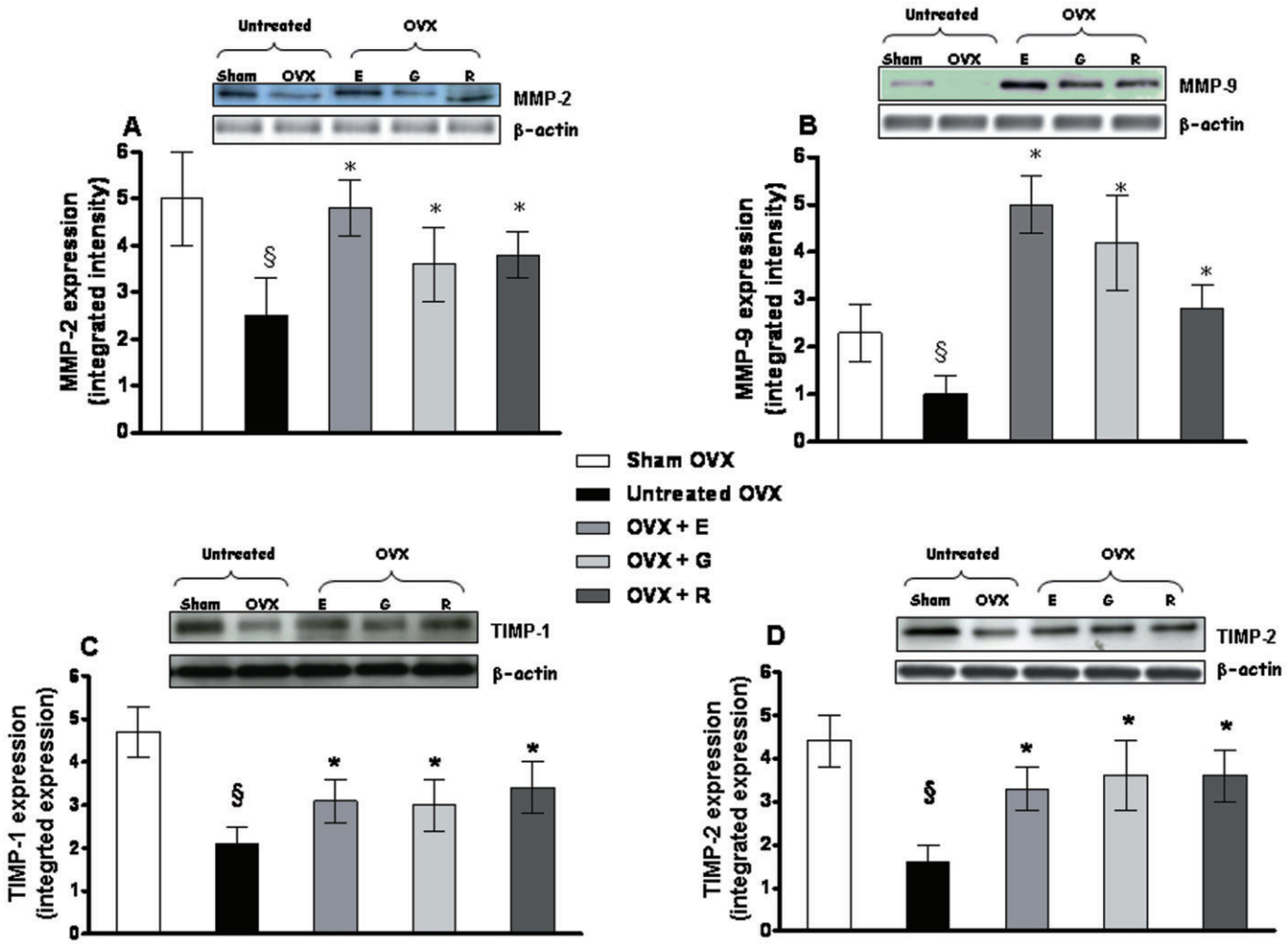

Figure 4

Representative Western blot analysis of MMP-2 (A), MMP-9 (B), TIMP-1 (C) and TIMP-2 (D) in skin biopsies from OVX rats treated, daily, with 17-α-ethinyloestradiol (E; 0.003 mg·kg^{-1} s.c.); genistein (G; 1 mg·kg^{-1} s.c.); raloxifene hydrochloride (R; 0.05 mg·kg^{-1} s.c.). The upper panel shows a representative autoradiography highlighting MMPs, TIMPs and β-actin (control) expression. The columns show quantitative data and represent the mean ± SD of six animals. $^{§}P < 0.001$ vs. sham OVX; $^{*}P < 0.001$ vs. untreated OVX.

animals. Overall, our data revealed that both oestradiol and genistein are more effective than raloxifene in improving these parameters, indicating the crucial role of ER selectivity (Shang and Brown, 2002). Recently, it has been reported that sustained exposure to oestrogen markedly delays wound re-epithelialization in mice, suggesting that these detrimental effects on healing could be related to ER-α (Gilliver *et al.*, 2010). In contrast, systemic treatment with genistein has been found to accelerate wound healing in OVX rats (Marini *et al.*, 2010b). Notably, 17 β-oestradiol displays relatively equivalent binding on both ER subtypes, and raloxifene binds with greater affinity to ER-α, but genistein aglycone binds preferentially to ER-β over ER-α, from 7- to 48-fold, in an oestrogen-deprived environment, depending on the assay system used (Kuiper *et al.*, 1997, 1998; Hsieh *et al.*, 2006). This latter effect is responsible for the peculiar mode of action of genistein: at therapeutic concentrations it binds preferentially to ER-β, which in turn down-regulates ER-α leading to the beneficial effects obtained via this isoflavone. However, Emmerson *et al.* (2010) demonstrated that some of genistein's effects on the skin are mediated by non-classical ERs. Specifically, genistein accelerates keratinocyte migration and re-epithelialization via insulin growth factor-1 receptor and its anti-inflammatory activity is likely to be attributable to an effect mediated via the MAPK pathway. Moreover, genistein also affects, via a non-genomic mechanism, cAMP signalling (Liu *et al.*, 2005) and/or the expression of longevity-associated genes such as glutathione peroxidase and Mn-superoxide dismutase (Mahn *et al.*, 2005; Borras *et al.*, 2006). The lowest genistein dose we used was surprisingly more effective in restoring skin properties than raloxifene or oestradiol. This finding was further supported by its ability to improve cell migration, inflammation, provisional matrix synthesis, collagen deposition, angiogenesis and re-epithelialization, all crucial processes in skin repair. At higher concentrations, genistein binds to ER-α, which could

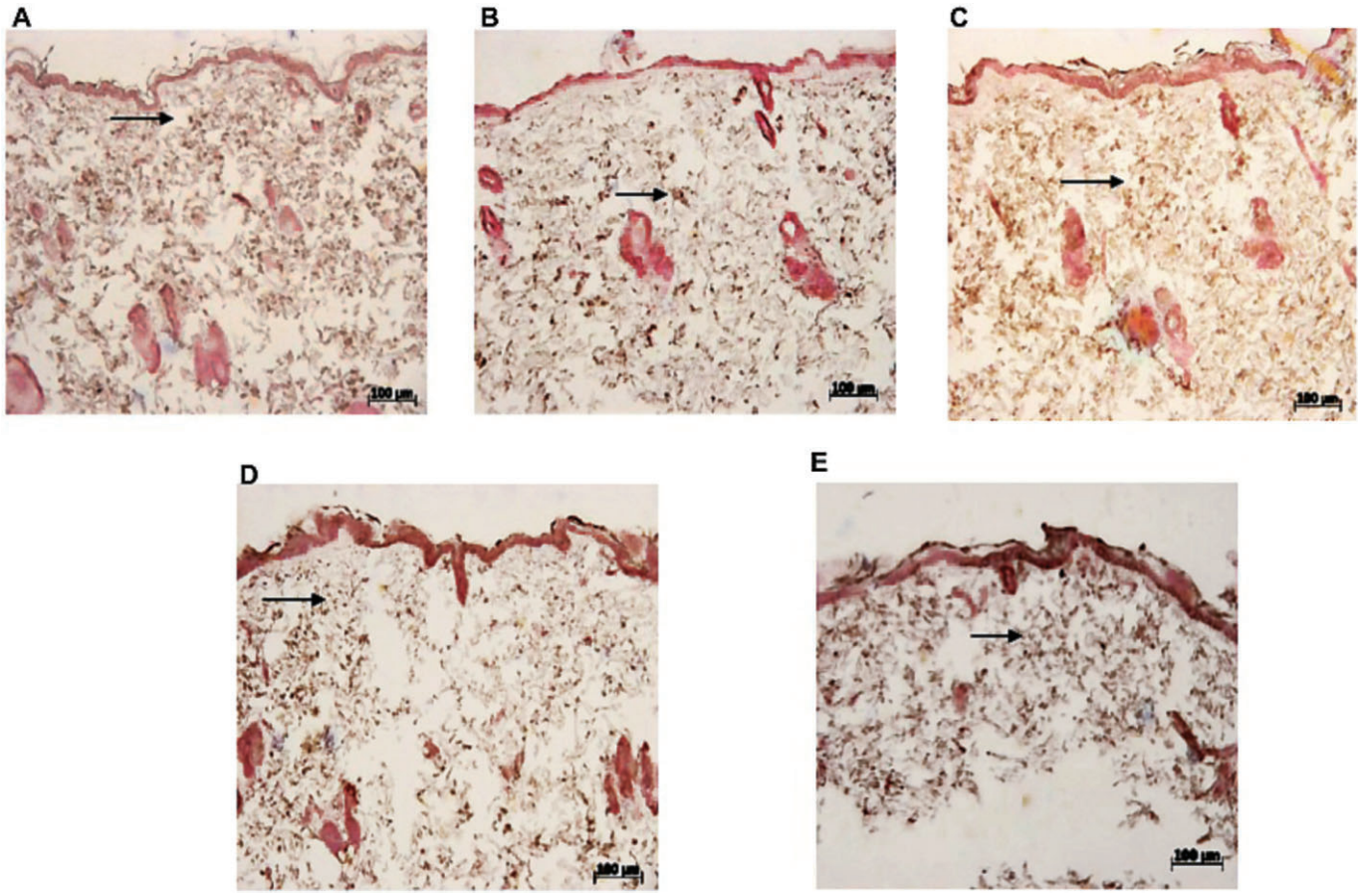

Figure 5

Light microscopy of MMP-2 immunostaining in skin biopsies from sham OVX (A), untreated OVX (B), OVX + 17-α-ethinyloestradiol at 0.003 mg·kg^{-1} s.c. (C), OVX + genistein at 1 mg·kg^{-1} s.c. (D) and raloxifene hydrochloride at 0.05 mg·kg^{-1} s.c. (E). Original magnification ×5. Arrows point to positively stained cells in dermal layer.

affect its overall positive effects obtained through ER-β binding. This is one hypothesis that may explain the better effects obtained with the lower genistein dose in our experimental model. Genistein could also induce other multiple non-genomic effects on the skin, which are dependent on its concentration, as have been previously observed in other tissues (Atmaca *et al.*, 2008).

Molecular data regarding TGF-β1 expression obtained in our experiments are encouraging and are well correlated with the histological parameters obtained earlier, suggesting that the increase in collagen thickness could be mediated by TGF-β1-producing cell types. Indeed, TGF-β1, a growth factor that stimulates fibroblast proliferation and ECM secretion, affects angiogenesis and epithelialization in the skin; in fact, it has been proposed that epithelialization is the main mechanism by which topical oestrogen increases ECM secretion (Son *et al.*, 2005). Moreover, as stimulation of ER-β results in enhanced keratinocyte proliferation (Merlo *et al.*, 2009) and migration, the augmented TGF-β1 levels might also originate from a prolonged stimulation of these cells as our experiment lasted for a relatively long period of time. An increase in VEGF expression is commonly observed with oestrogen therapy, while, to date, the effect of genistein and raloxifene on VEGF has not been elucidated. However, in our experimental model we found an increased content of VEGF in skin samples following s.c. administration of genistein or raloxifene. In fact, it has recently been shown that TGF-β1 induces angiogenesis through VEGF-mediated apoptosis (Ferrari *et al.*, 2009), supporting our experimental observations. This finding is interesting as VEGF has been shown to have a pivotal role in the initiation of angiogenesis, based on its ability to induce the expression of proteases that digest ECM components (Chung and Eun, 2007). Hence, it is possible that molecular cross-talk occurs whereby TGF-β, by increasing collagen thickness, is also responsible for an augmented need for oxygen, which in turn leads to an increase in VEGF. Degradation of ECM, which is mediated by MMPs, is also critical for embryonic development and adult tissue homeostasis (Birkedal-Hansen, 1995; Werb and Chin, 1998; Chung and Eun, 2007). Conversely, uncontrolled ECM degradation is associated with pathological processes such as autoimmune skin blistering diseases and tumour invasion (Parks, 1999). Specifically, MMPs are a family of zinc-containing proteinases that have the ability to degrade most ECM (Kahari and Saarialho-Kere, 1999). Among the MMP family there are two unique members, MMP-2 and MMP-9, both of which contain

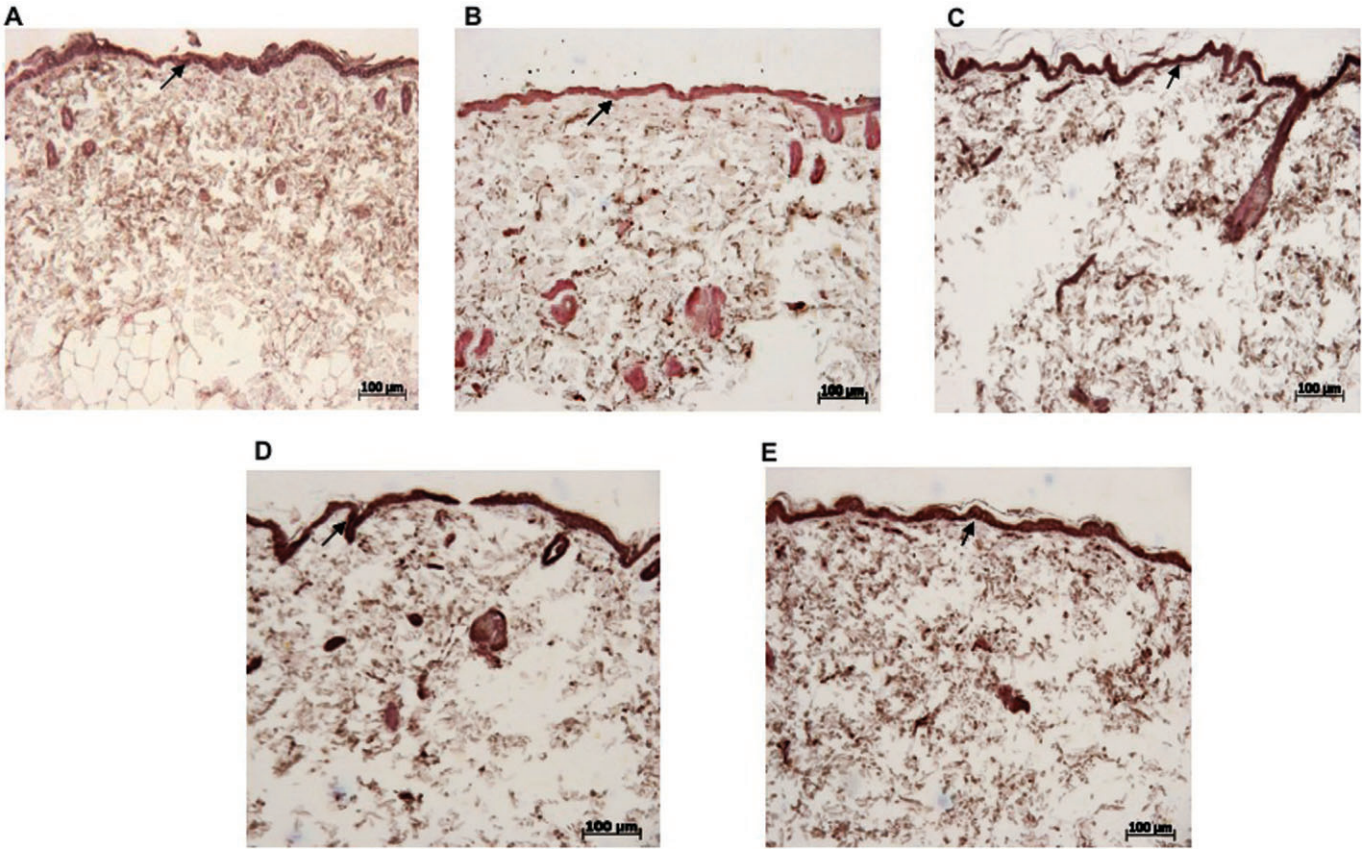

Figure 6

Light microscopy of MMP-9 immunostaining in skin biopsies from sham OVX (A), untreated OVX (B), OVX + 17-α-ethinyloestradiol 0.003 mg·kg^{-1} s.c. (C), OVX + genistein 1 mg·kg^{-1} s.c. (D) and raloxifene hydrochloride 0.05 mg·kg^{-1} s.c. (E). Original magnification ×5. Arrows point to positively stained cells in epidermal layer.

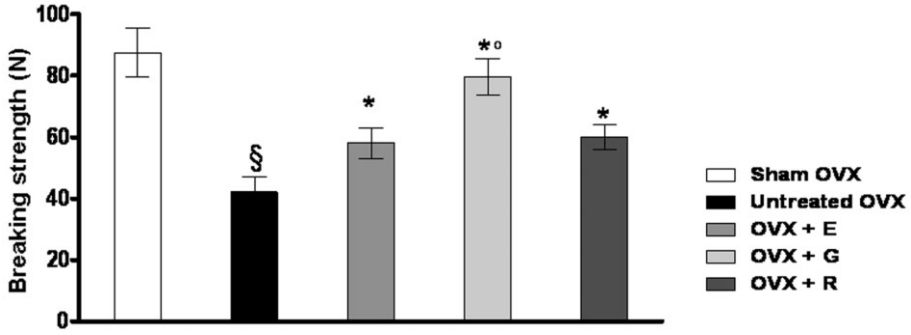

Figure 7

Breaking strength of skin biopsies from ovariectomized rats (OVX) treated, daily, with 17-α-ethinyloestradiol (E; 0.003 mg·kg^{-1} s.c.); genistein (G; 1 mg·kg^{-1} s.c.); raloxifene hydrochloride (R; 0.05 mg·kg^{-1} s.c.). The columns show quantitative data and represent the mean ± SD of six animals. $^{§}P < 0.001$ vs. sham OVX; $^{*}P < 0.001$ vs. untreated OVX; $^{°}P < 0.001$ vs. OVX + raloxifene; $^{°}P < 0.001$ vs. OVX + oestradiol.

fibronectin-like domains for collagen binding (Birkedal-Hansen *et al.*, 1993). Accumulating evidence demonstrates that activation of these enzymes is controlled by specific TIMPs (Murphy *et al.*, 1999a,b). Although many factors including cytokines, growth factors and ECM proteins have been reported to promote the expression of TIMPs, it is not known whether these factors work together or antagonistically in human tissue. However, upon injury such as mechanical trauma, thermal burn and viral infection, MMP-2 and MMP-9 are promptly elevated, indicating their role in skin reparation. Data obtained by Western blot and immunohistochemical analysis in our OVX rats indicated that

genistein significantly raised MMP-2 and MMP-9 levels, suggesting an increased reparative action on skin. On the other hand, through an increase in the respective TIMPs, genistein also prevented uncontrolled ECM degradation. Similarly, oestradiol, and to a lesser extent raloxifene, exerted a comparable action restoring ovariectomy-impaired skin and ECM protein balance. In agreement with the histological and molecular data, genistein also re-established the mechanical properties of the skin, as demonstrated by its ability to significantly enhance the breaking strength of the skin of OVX animals.

Interestingly, our experimental data also indicated that systemic administration of genistein is able to produce positive effects on ovariectomy-induced skin changes in the same dose range as that used in postmenopausal women (Squadrito *et al.*, 2003; Atteritano *et al.*, 2007; Marini *et al.*, 2007, 2008a,b, 2010a; D'Anna *et al.*, 2009).

The safety of ER modulating compounds is always a concern, especially in post-menopausal women as they age (North American Menopause Society, 2010; Santen *et al.*, 2010). Though pure genistein has been shown in an athymic nude mouse to induce growth of an implanted human cancer cell line (Ju *et al.*, 2002, 2006), the preponderance of evidence demonstrates that genistein in humans has a low risk of inducing reproductive cancer and may be protective against neoplastic formation (Taylor *et al.*, 2009).

In conclusion, our results suggest that genistein might be a useful alternative to oestrogen for the management of age-related skin changes commonly observed in postmenopausal women. However, further studies are needed to compare the effectiveness of different routes of administration and to establish the parameters for the use of this molecule in skin remodelling and ageing.

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Conflicts of interest

All the authors have none to declare.

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