

Research Article

In An Incisional Model of Wound Healing, Genistein Aglycone Enhances Skin Recovery: A Comparison with Raloxifene and Estradiol in Ovariectomized Rats Is Presented

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Abstract

Context and goal: Poor wound healing is commonly associated with estrogen depletion during menopause. Experiments on anti-aging cosmetic formulations using genistein have yielded intriguing findings about skin health. Here, we examined the effects of systemically administered the genistein aglycones in an incisional wound healing model in comparison to systemically administered estradiol and raloxifene. Method of experimentation: Rats were randomly divided into groups of 12 animals each six months after Ovariectomies (OVX) and given daily treatments of raloxifene hydrochloride (0.05 and 0.5 mg·kg⁻¹s.c.), genistein aglycone (1 and 10 mg·kg⁻¹s.c.) or 17- α -ethinyl estradiol (0.003 and 0.03 mg·kg⁻¹s.c.) for a period of 12 weeks. Rats with OVX and sham OVX were not treated and served as controls. Then, an incisional wound healing technique was carried out 14 or 7 days before to the experiment's conclusion and skin specimens were gathered to assess molecular, histological and functional measurements. Important Results: Compared to samples from sham OVX animals, samples from OVX rats seven and fourteen days after wounding shown a decrease in transforming growthfactor-b1, tissue transglutaminase 2 and vascular endothelial growth factor. Genistein, raloxifene and estradiol all considerably altered this decline, but the lowest dose of genistein had a stronger impact than the other two therapies. Furthermore, the best genistein dosage for enhancing wound tensile strength and skin healing was the lowest one. Inferences and conclusions: One potential alternative treatment for the control of skin wound healing is genistein aglycone.

Keywords: Wound Healing; Skin; Menopause; Rats; Genistein Aglycone; Estrogen; Raloxifene

Abbreviations:

ER: Estrogen Receptor; HRT: Hormone Replacement Therapy; OVX: Ovariectomized; SERMs: Selective Estrogen Receptor Modulators; TG2: Tissue Transglutaminase 2; TGF-b1: Transforming Growth Factor-b1; VEGF: Vascular Endothelial Growth Factor

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Introduction

Numerous experimental and clinical investigations have demonstrated that the loss of estrogen during menopause is often accompanied by skin shrinkage, a decrease in collagen and water content, a loss of elasticity and hyperandrogenism symptoms [1,2]. Moreover, it is believed that the cumulative effects of estrogen insufficiency on skin have a role in the poor wound healing that comes with human aging [3]. One of these detrimental impacts on cutaneous wound healing is an augmented vulnerability to trauma, leading to delicate skin that rips and bruises readily. In general, little is known about the precise role that estrogens play in wound healing and the metabolic pathways that lead to wound repair. Reduced collagen synthesis, elevated elastase and low levels of Transforming Growth Factor-b1 (TGF-b1) have all been linked to age-related delays in wound healing [4-6]. Thus, Research has demonstrated that estrogens promote healing by causing dermal fibroblasts to secrete TGF-b1 [6]. Even though a

direct correlation between estrogen therapy and the inflammatory phase of wound healing needs more research, decreased oestrogen levels have also been linked to impaired cytokine signal transduction in the inflammatory phase of wound repair [7]. In the wounds of Ovariectomized (OVX) rats, estrogen treatment has also been demonstrated to increase collagen deposition [8]. However, systemic oestrogen either has no effect or decreases collagen deposition, depending on the dose and amount of time since wounding [9-11]. Studies conducted on animals typically yield conflicting results on the impact of estrogen levels on the various stages of wound healing. These disparities most likely result from variations in species, length of therapy and techniques used [12]. Hormone Replacement Treatment (HRT) has been widely used to treat menopausal symptoms, it also appears to greatly accelerate wound healing [6,13-15]. In this regard, topical oestrogen, as opposed to a placebo, decreases the activity of elastase in skin wounds, according to a randomized, double-blind trial conducted on aged men and women [16]. However, there are currently relatively few overall data on human wound healing, necessitating more research before any firm suggestions can be made. In fact, certain women's beliefs of the risks and side effects of estrogen reduce the therapeutic value of hormone replacement therapy, which in turn limits its potential application to a larger range of people. As a result, in recent years, several alternative treatment approaches have had to be taken into consideration. Lately, through particular Estrogen Receptor (ER) interaction, Selective Estrogen Receptor Modulators (SERMs) were developed in an effort to maximize the positive benefits of estrogen while limiting the negative side effects in target tissues. Comparing raloxifene to estradiol, one of the most researched SERMs, has been shown to improve wound healing in OVX mice [17]. Additionally thought to be a natural SERM, phyto estrogen genistein may help prevent poor wound healing without having negative estrogenic side effects on breast and uterine tissue [18]. In particular, isoflavone-containing cosmetic treatments have been used to reduce wrinkles and dryness in the skin and genistein aglycone has consistently demonstrated success in controlling circumstances of estrogen deficiency [19]. Previous experimental and clinical studies conducted by our research group have already shown the effectiveness and safety of genistein aglycone in stronger genistein affinity for the ER-b than the ER-a in a low-oestrogen environment and this promising profile may be a direct result of this [20-27]. Interestingly, ER-bis is more broadly distributed in the skin and skin structures [28]. In addition, oestradiol up-regulates ER-b receptors in keratinocytes at physiological concentrations, encouraging proliferation [29]. Human skin fibroblasts are similarly targeted by estrogens and since ER-a and ER-b co-express in these cells, it is highly probable that ER ligands, like genistein, also regulate the extracellular matrix in the skin [30]. The current study's parent experiment, which used OVX rats administered with genistein aglycone or oestradiol raloxifene, showed that the isoflavone genistein can restore osteoporosis brought on by oestrogen deficiency [31]. We created an incisional wound in these same animals to assess the healing potential of a prolonged course of treatment with the aforementioned chemicals. These studies' findings have made it easier to understand the intricate molecular interactions that occur between skin and phyto-oestrogens throughout menopause and the potential for averting the cellular and molecular alterations that impair appropriate wound healing in postmenopausal women (Fig. 1).

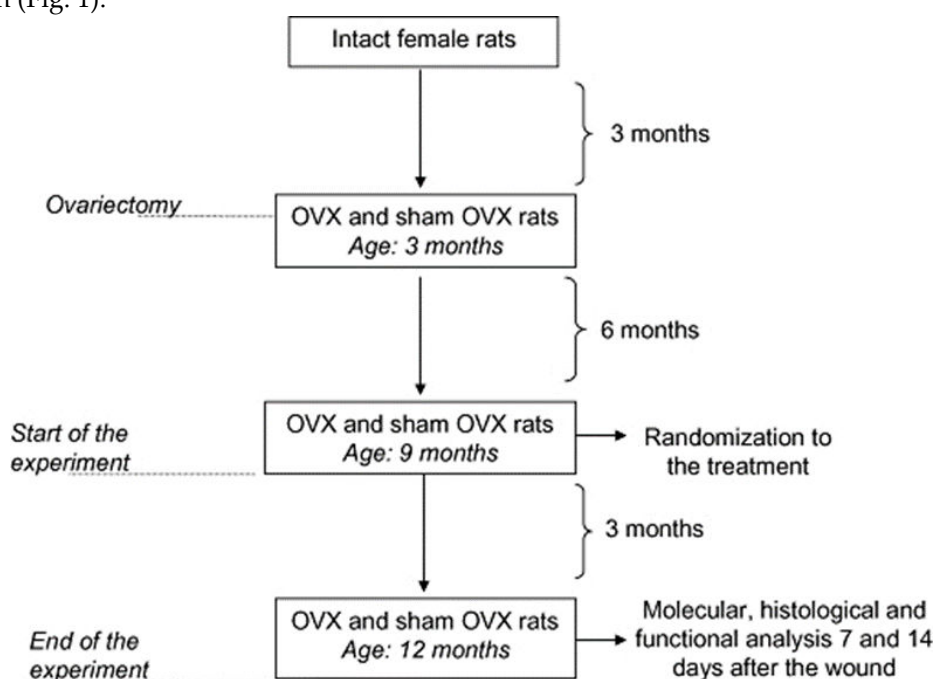


Figure 1: Scheme of the experimental protocol.

Methods

The animals were kept in the animal facility of during the studies, fed standard laboratory animal diet and given access to unlimited water and kept in controlled ambient conditions with a 12-hour light-dark cycle and a temperature of roughly 24°C. In summary, a total of 84 female Spaug-Dawley rats (Charles River, Calco, Italy) weighing between 250 and 275 g at 12 weeks of age were bought, along with 12 sham OVX rats. The animals were split up into eight groups of twelve animals each after six months. Untreated OVX rats were a group of rats that were not given any treatment. As controls, there were two groups: the fake OVX group and the untreated OVX group. For a period of 12 weeks, subcutaneous daily administration of the various treatments genistein aglycone (1 and 10 mg·kg⁻¹), 17- α -ethinyloestradiol (0.003 and 0.03 mg·kg⁻¹) and raloxifene hydrochloride (0.05 and 0.5 mg·kg⁻¹) was performed. Anesthesia overdose claimed the lives of the animals. The injury was sustained on the back of every animal, as previously mentioned, seven or fourteen days before to slaughter [32]. The rats were put under deep ether anesthesia, their back hair was shaved, their skin was cleaned with a povidone-iodine solution and sterile water was used to wipe it all out. On the rat's dorsum, two full-thickness longitudinal incisions (4 cm) were created. 4-0 silk surgical sutures were used to close the wound edges at intervals of 1 cm. Using a scalpel, an ellipse-shaped cut was made around the lesion to remove the skin. Following the experiment, the wounds were extracted, separated into three sections and their breaking strength was measured on day 14. Only day 14 of the center strip was used for histology, while the remaining segments were used for molecular analysis. Uninjured skin specimens, utilized for biopsies from either untreated sham or OVX animals were taken for comparisons on the days of the wounding procedures. TGF- β 1 and tissue Transglutaminase 2 (TG2) were measured by Western blot analysis. In summary, skin samples were homogenized using an Ultra-Turrax homogenizer (IKA Company, Staufen, Germany) in 1 mL of 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 methyl sulphonyl fluoride, aprotinin, leupeptin, pepstatin A (10 mg·mL⁻¹ each) and 100 mM Na³VO₄). Centrifugation was applied to the homogenate for 15 minutes at 15,000 rpm. Supernatant was obtained and utilized with the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA) for protein measurement. A 30 mg protein sample was denatured in reduction buffer (62 mM Tris/HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β -mercaptothion, 0.003% bromophenol blue), then separated using an SDS (12%) electrophoresis gel made of polyacrylamide. Proteins were loaded onto a nitrocellulose membrane at 100 mA for one hour using the transfer buffer (48 mM Tris, 20% methanol and 39 mM glycine). After confirming that the amounts of protein were equal, membranes were stained with Ponceau S (0.005% in 1% acetic acid), blocked with 5% non-fat dry milk in TBS-0.1% Tween for one hour at room temperature and incubated with a primary antibody for TGF- β 1 (Upstate, NY, USA) and TG2 (Cell Signaling, Beverly, MA, USA) in TBS-0.1% Tween overnight at 4°C, diluted 1:500. The membranes were incubated with a secondary antibody peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pierce, Rockford, IL, USA) for 1 hour at room temperature diluted 1:20,000 after being cleaned three times for ten minutes each in TBS-0.1% Tween. Following washing, the enhanced chemiluminescence technique was used to analyze the membranes in accordance with the instructions provided by the manufacturer (Amersham, Little Chalfont, UK). Via scanning densitometry, the protein signal was measured using a bio-image analysis system (Bio-Profil Celbio, Milan, Italy). By using a rabbit monoclonal antibody (Cell Signaling) diluted 1:500 and peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pierce) diluted 1:15,000 to detect β -actin, the equal loading of protein was evaluated on stripped blots. Purification of all antibodies was achieved using peptide affinity chromatography and protein A. Vascular Endothelial Growth Factor (VEGF) in wound analysis. In summary, 1.0 milliliter of 1% PBS containing the Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN, USA) was used to homogenize the tissues. After centrifuging homogenates to remove debris, they were filtered using a 1.2 μ m porous syringe filter. A commercially available human VEGF-specific enzyme-linked immunosorbent test kit was used for the analysis. The VEGF concentration was given as pg stands for ng protein. As previously mentioned, a calibrated tensometer (Sans, Milan, Italy) was used to assess the maximum load (breaking strength) that wounds could withstand on coded samples without knowledge of the treatments [32]. Breaking strength was defined as the mean highest level of tensile strength in Newtons (N) prior to the separation of wounds. The ends of the skin strip were tugged at a constant speed of 20 cm·min⁻¹. Histological assessment Skin samples were preserved in 10% buffered formalin in order to be examined under a light microscope. Following fixation, sections that were perpendicular to the wound's anterior-posterior axis were graded with ethanol and embedded in paraffin. Sections (5 mm thick) were placed on glass slides, rehydrated in distilled water and then stained with Masson's trichrome or hematoxylin and eosin. A pathologist inspected each slide as part of the histological evaluation without being aware of the prior procedure, which involved utilizing masked slides under a microscope with a magnification of $\times$ 5 to $\times$ 40. The following criteria were assessed: angiogenesis, granulation tissue development and epidermal and dermal healing. For the purpose of grading, the margins of the wound in each segment and typical control wounds were compared (Table 1). Only

mature vasculature containing erythrocytes were counted in relation to angiogenesis. The following factors were taken into account in order to distinguish between well-formed and poorly-formed capillary vessels: the existence or absence of oedema, congestion, bleeding, thrombosis and intravascular or intervascular fibrin development. The data pertaining to wound healing in experimental models were used to evaluate the histological score used in this investigation [32]. Analytical statistics Means and SD are used to express all data. Tukey's multiple comparison test was used after one-way ANOVA to analyze comparisons between various treatments. A probability of less than 0.05 was chosen as the threshold in each situation (Fig. 2,3).

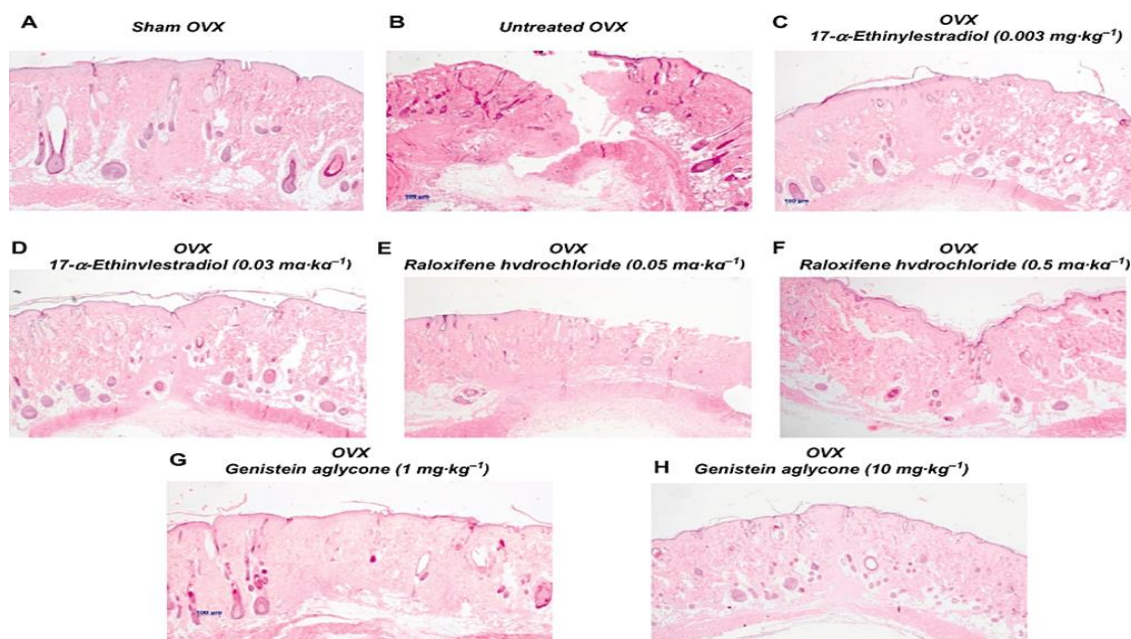


Figure 2: (A-H) Light microscopy of incisional wounds in OVX rats: effects of 17- α -ethinyloestradiol (0.003 and 0.03 mg·kg⁻¹ s.c.), raloxifene hydrochloride (0.05 and 0.5 mg·kg⁻¹ s.c.) and genistein aglycone (1 and 10 mg·kg⁻¹ s.c.); (haematoxylin and eosin, $\times 5$ original magnification).

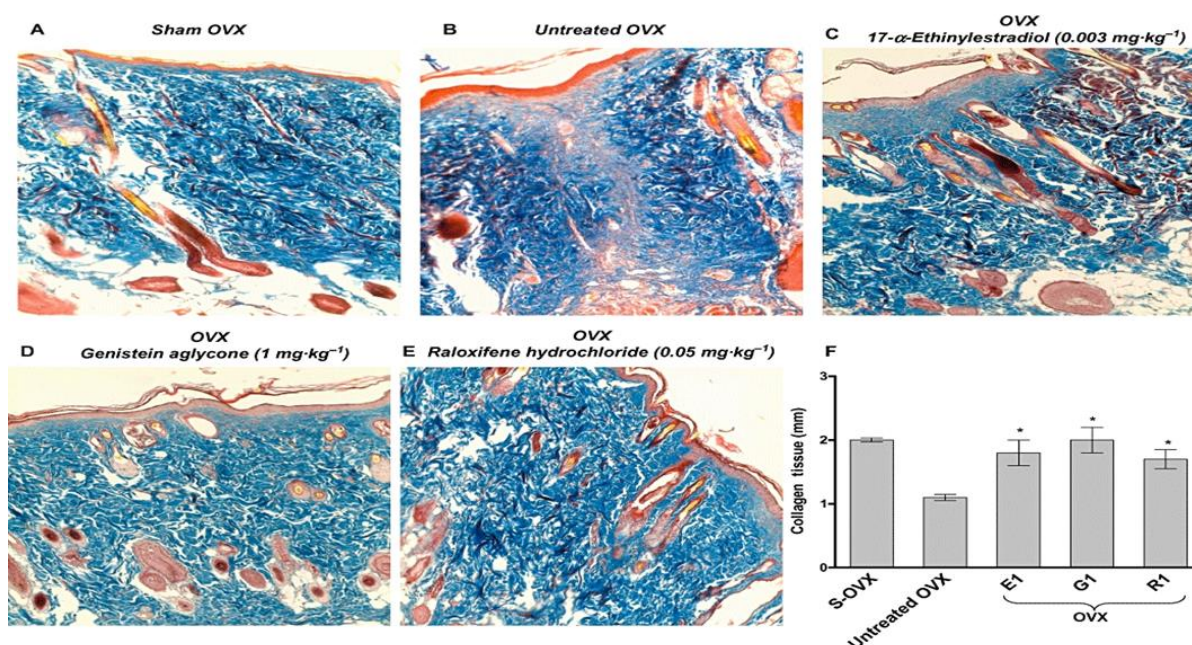


Figure 3: (A-E) Light microscopy of incisional wounds in OVX rats (Masson's trichrome stain, $\times 10$ original magnification) and (F) quantification of collagen tissue. Representative data describing collagen tissue thickness are shown as the mean $\pm$ SD of six animals. *P < 0.001 versus untreated OVX.

Discussion

Reduced systemic estrogen production such as that experienced by women going through menopause or having an ovariectomy delays wound healing, increases local inflammation and decreases collagen deposition. HRT or topical 17 β -oestradiol administration can correct these defects in humans nevertheless, not all menopausal women should receive HRT due to these and other positive effects of oestrogens [1,2,6,33]. Moreover, as concentration and application sites need to be carefully monitored to prevent systemic side effects and respect skin physiology, topical oestrogen therapy for skin diseases must be provided by a qualified dermatologist. Given this context, the use of SERMs and other alternative therapy modalities has been explored as a means of treating or preventing the negative effects of oestrogen deficiency. In particular, a lot of focus has been on certain phyto-oestrogens. Particular isoflavones can distinguish between desirable and undesired biological consequences due to their tissue-specific oestrogen activities [34,35]. Since most studies on skin healing characteristics have focused on acute therapies that are initiated right away after wounding, we decided to look at the long-term effects of various treatments in an incisional wound healing model in the current experiment. Here, we showed for the first time that long-term administration of the isoflavone genistein aglycones might improve extracellular matrix remodeling and turn-over in OVX rats by preventing delayed wound healing. Furthermore, on all skin parameters examined at days 7 and 14 after wounding, genistein aglycone was found to be more effective than oestradiol or raloxifene. In this case, molecular information about TGF- β 1 and TG2 expression in OVX rat wounds used in Our test results are positive. While TGF- β 1 can have contradictory effects on the skin, it is widely recognized that this mediator, which is released by cells found at tissue repair sites, including platelets, activated macrophages and possibly fibroblasts, has positive effects at every stage of the wound healing process [36,37]. TGF- β 1 is thus required for appropriate monocyte chemoattraction during the inflammatory phase; during the proliferative phase, it increases the synthesis of extracellular matrix and influences angiogenesis and epithelialization; ultimately, it produces contraction and myofibroblast development in skin wounds. TGF- β 1 has been shown to accelerate healing and strengthen the breaking strength of the repaired tissue in older animals. It also promotes angiogenesis, which increases blood flow to dermal wounds, in part by inducing the local release of other growth factors [38]. According to an *in vitro* study, activation of the ER- β was adequate to achieve a stimulatory effect on wound healing via a method that did not necessitate overproduction of TGF- β 1 [29]. Our findings, however, only demonstrate a rise in TGF- β 1 in response to genistein aglycone injection when compared to untreated OVX rats not to sham OVX animals. Given that our experiment lasted for a considerable amount of time and that ER stimulation leads to increased keratinocyte proliferation and migration, the increased TGF- β 1 levels may have resulted from prolonged stimulation of these cells. It would be interesting to confirm this theory in an excisional wound healing model in this particular circumstance. In actuality, an excisional wound healing model would help us comprehend whether genisteinicallycone can likewise differentiate fibroblast into myofibroblasts by means of TGF- β 1 and/or other non-ER-mediated pathways, hence facilitating an appropriate wound contraction. in the stage of maturation. Of course, part of our future research will involve more experiments to elucidate this method of action. According to Griffin, et al., TG2 has significant effects on the extracellular matrix that are both direct and indirect [39]. Direct effects include protein cross-linking, which stabilizes the matrix and indirect effects include TGF- β 1 activation, which causes matrix deposition. When coupled to fibronectin, TG2 can also function independently as a cell adhesion protein, reducing anoikis-induced cell death [40]. Histological score and increased breaking strength in genistein-treated OVX rats show that the ultimate consequence is an acceptable wound healing and maintenance of tissue integrity. It was surprising to learn that genistein aglycone improved skin trophism as good as or better than other well used treatments. TGF- β 1 and TG2 stimulation after genistein aglycone injection may additionally explain why wounds have a higher VEGF concentration. This is an intriguing finding: according to Bao, et al., VEGF plays a crucial role in the start of angiogenesis by promoting the expression of proteases that break down extracellular matrix components that obstruct angiogenesis, endothelial cell proliferation and apoptosis prevention [41]. In fact, neutrophils, macrophages and fibroblasts all frequently produce VEGF during normal healing; however, in conditions such as oestrogen loss and/or aging, VEGF dysregulation may hinder healing by lowering the inflammatory response and the quantity of fibroblasts in the skin. In this regard, our findings are in complete agreement with earlier research demonstrating that estrogens promote wound healing and raise VEGF in a variety of tissues [42,43]. The observed chemical effects in this experimental setup are brought about by persistent Each histological criterion that was taken into consideration, such as epidermal regeneration, granulation tissue thickness, fibroblast creation and the development of new, well-structured capillary vessels, showed a strong correlation with genistein aglycone treatment. It was found that, 14 days after wounding, the lowest dose of genistein was superior to either oestradiol or raloxifene hydrochloride in terms of speeding wound healing. This latter discovery provides additional evidence that the processes of skin repair enhanced by genistein administration include cell migration, inflammation, provisional matrix production, collagen deposition, angiogenesis and re-epithelialization.

Additionally, all treatments restored the thickness of the collagen layer following wounding, but genistein aglycone was the only treatment that clearly also re-established the altered architecture of the collagen tissue, which was found to be significantly reduced by oestrogen loss, ageing and wounding in untreated OVX animals. In conclusion, genistein aglycone proved to be the most advantageous treatment with regards to skin functionality. It frequently shown that alterations in skin collagen cause a decrease in skin strength and suppleness in menopausal women. In actuality, rats given subcutaneous genistein injections had incisional wounds with greater breaking strengths than rats given raloxifene hydrochloride or oestradiol. This latter suggests that genistein can also heal the mechanical qualities of injured skin through its various activities. According to our experimental results, two doses of genistein aglycone administered subcutaneously to OVX rats consistently were found to have favorable effects on wound healing within the same dose range as when post-menopausal women received oral treatment with 54 mg of aglycone genistein per day [21-24,27]. Thus far, the theory that the current experimental results could be connected to Genistein's acute effect can be ruled out with ease because, in earlier studies (data not shown), we did not find that administering genistein to OVX rats for 14 days after wounding had any discernible effect. These encouraging results after systemic genistein aglycone treatment are most likely due to selective ER-b activation, specifically in the stratum basalis, stratum spinosum, stratum granulosum and papillae dermis of OVX rat skin [45]. In actuality, genistein aglycone binds preferentially to ER-b over ER-a in an oestrogen-deprived environment and this selectivity was 7- to 48-fold, depending on the assay system employed. This is in contrast to 17 β -oestradiol, which exhibits relatively equal binding to both ER subtypes and raloxifene, which binds with greater affinity to the ER-a. Our experimental Data also demonstrate the need for appropriately titrating the concentration of genistein aglycone, since some reports imply that at low doses, an inadequate stimulation of ER-b may occur, resulting in few or nonexistent beneficial effects [46-50]. In fact, genistein aglycone binds to ER-a at larger quantities and as has been shown in the past in other target tissues (bone, endothelium), this may reduce the overall beneficial benefits on wound healing that ER-b-binding provides. It is also important to emphasize that genistein's effects on skin wound healing may be connected to a variety of non-genomic actions on skin cells, as has been shown in other target tissues (Fig. 4,5) [51-59].

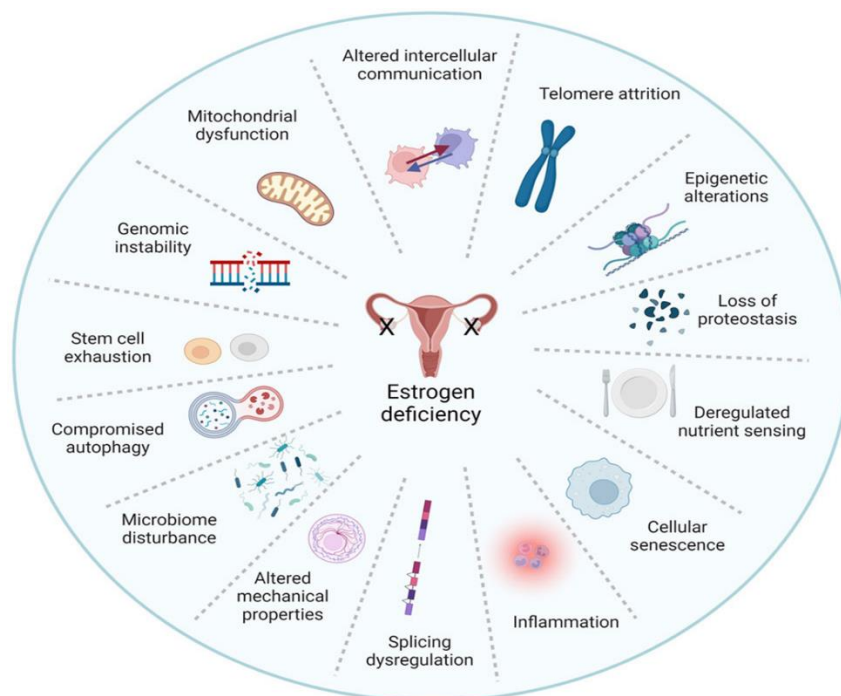


Figure 4: Estrogen is a pleiotropic factor in the hallmarks of aging. The pillars of aging as described by Kennedy, et al., and Schmauk-Medina, et al., are highly intertwined processes and understanding the interplay between these factors is critical. Remarkably, existing literature has linked estrogen signaling to virtually all hallmarks of aging. Herein we propose that estrogen deficiency plays a major role in skin aging biology.

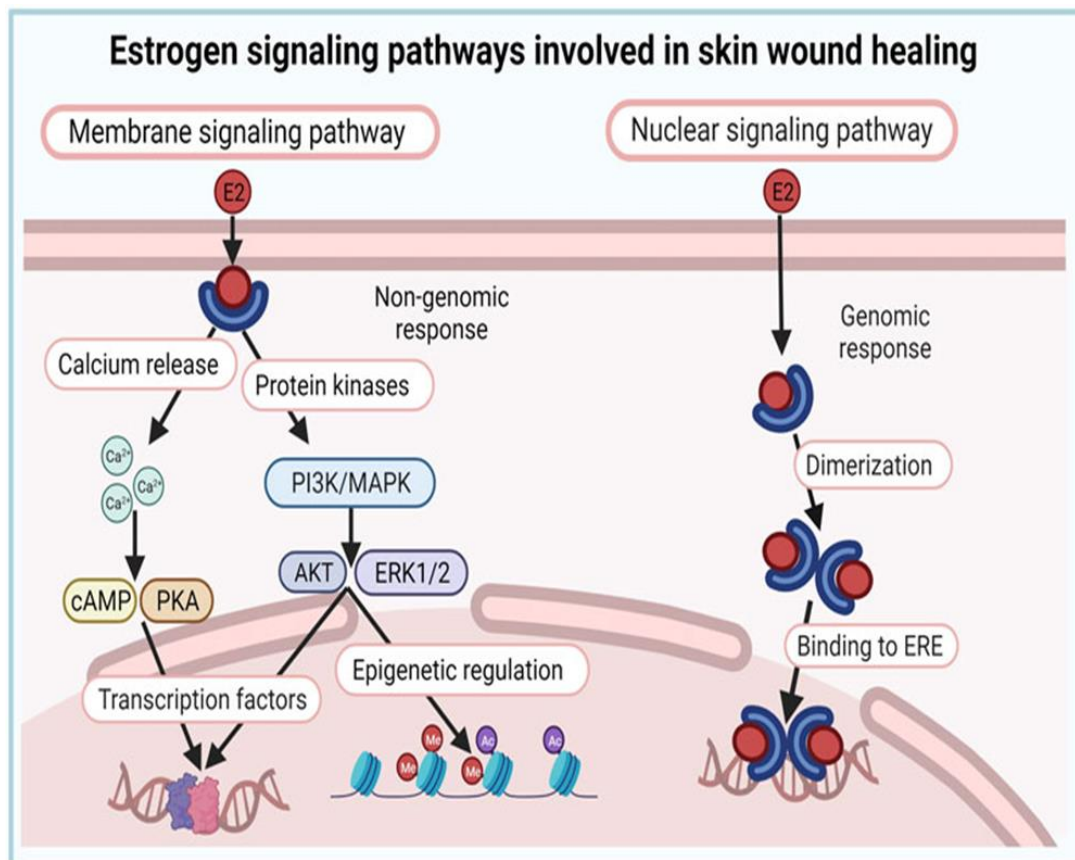


Figure 5: Estrogen signaling pathways involved in skin wound healing. Estrogen effects in the skin are likely mediated through both genomic (nuclear) and non-genomic (membrane) signaling pathways.

Conclusion

All things considered; our findings strongly imply that genistein aglycone may be a novel prospective treatment for the management of post-menopausal women's skin wound healing processes. To determine whether genistein aglycone might also offer a novel therapeutic method for treating additional human dermatological problems, more research as well as a comparison between topical and systemic administration of the drug are necessary.

Conflict of Interest

The author has no conflict of interest to declare.

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