ORIGINAL ARTICLE

Wound Healing Properties of Phytoestrogens: *In Vitro* and *In Vivo* Studies

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ABSTRACT

Introduction: Phytoestrogens are compounds derived from food that resemble mammalian estrogen structurally as well as mimic the estrogenic activity. The capability of phytoestrogen to bind to estrogen receptor owes to its phenolic ring, which presents together with two hydroxyl groups. While previous studies showed that estrogen accelerates healing in older women, there is a lack of understanding of the effect of phytoestrogens in the context of wound healing. Hence this study aims to investigate the potential of phytoestrogens in stimulating the recovery of skin injury. **Methods:** Apigenin, luteolin, chrysin, quercetin and kaempferol were investigated for their ability to close the gap in a scratch wound assay. Three most potential phytoestrogens from the *in vitro* experiment were selected and given to wounded ovariectomized mice. **Results:** Apigenin, luteolin and chrysin were found to induce human dermal fibroblasts to migrate into the pseudo-wound area comparable to estrogen ($p < 0.0001$), resulting in percentage wound closure of 67, 71 and 75, respectively. Following treatment with apigenin, luteolin and chrysin *in vivo*, chrysin-treated ovariectomized mice showed an accelerated wound healing compared to other candidates, associated with significant wound area reduction ($p < 0.001$), increased re-epithelialization ($p < 0.05$) and reduced macrophage infiltration ($p < 0.05$). **Conclusion:** Finding from the present study suggests chrysin as a prospective agent for the treatment of skin wound. Intake of chrysin-rich food is anticipated to promote recovery of chronic wound especially in people who has low level of estrogen such as in postmenopausal women and elderly women.

Keywords: Phytoestrogens, Flavonoids, Estrogen, Wound healing

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INTRODUCTION

Phytoestrogens are plant-derived compounds that resemble estrogen structurally and have similar physiological outcomes. Chemical structures resembling that of endogenous estrogen allow phytoestrogens to interact with estrogen receptors and thus exert estrogen-like actions (1). Phytoestrogens have been suggested to alleviate conditions associated with menopausal symptoms such as hot flushes either through high consumption of phytoestrogen in foods or by taking supplements (2,3). Phytoestrogen seems to exert significant impacts on bone formation and proposed to be cardioprotective compounds owing to their effects in reducing cholesterol levels in the plasma, delayed atherosclerosis and improved vascular functions (4,5).

Previous study has demonstrated an improvement in wound healing *in vitro* and in animal model following treatment with the soy-derived flavonoid, genistein (6). Genistein, belongs to the class of isoflavones, has been shown to enhance vascularization and proliferation in the dermis when utilized in topical form (7). Flavones and flavonols are additional subclasses of flavonoids, which have been researched widely for their biological potency (8). Apigenin, luteolin and chrysin are flavones while quercetin and kaempferol are classified as flavonols. There are slight structural differences between flavones and flavonols, with flavonols possessing a hydroxyl group at the C-3 position in C ring which is absent in flavones, and this may contribute to varied biological effects. Previously identified biological effects of apigenin, luteolin, chrysin, quercetin and kaempferol include ability to suppress inflammation and bacterial growth, to reduce cell damage by excessive oxidation and to trigger the synthesis of collagen for formation of new extracellular matrix which could assist in wound healing process (9-12).

It has been reported that decreased levels of estrogen may lead to poor wound repair, especially in the elderly (13). Studies have shown that estrogen administration could accelerate healing of wound in postmenopausal

women and animal model of ovariectomized mice (14,15). Compounds that resemble estrogen, such as phytoestrogen, could perhaps exert a comparable effect to estrogen in accelerating wound repair. However, there is a meagre understanding of whether phytoestrogens could exert similar effects as estrogen on skin wound healing. Hence this study aims to investigate the effects of a group of phytoestrogens; apigenin, chrysin, luteolin, kaempferol and quercetin in accelerating skin wound healing.

MATERIALS AND METHODS

Reagents and antibodies

Apigenin, luteolin, quercetin and kaempferol were purchased from Tocris Bioscience, UK, while chrysin was purchased from Sigma-Aldrich, UK. Apigenin, luteolin, quercetin, kaempferol and chrysin were dissolved in sterile-filtered dimethyl sulfoxide (DMSO; Sigma-Aldrich, UK). Final DMSO concentration was 0.1% in apigenin-, luteolin-, quercetin-, kaempferol-, chrysin- and vehicle- treated cells.

Cell culture

Primary human dermal fibroblasts were kindly provided by Rachel Crompton (University of Manchester, UK), which were obtained from the abdominal skin of a 38-year old female donor. Cells were cultured in phenol red-free media (Sigma-Aldrich, UK) containing 10% charcoal-stripped serum (Sigma-Aldrich, UK) and maintained at 37 °C, 5% CO_2.

Scratch wound assay

Primary human dermal fibroblasts were plated into a 24-well plate in phenol red-free media containing 10% charcoal-stripped serum to form a confluent cell monolayer. Prior to the scratch, cells were incubated with 4 µg/ml mitomycin C (Sigma Aldrich, UK) to prevent cell proliferation, thus ensuring that scratch closure was due to cell migration rather than increased cell number. A vertical scratch was created through the cell monolayer using a sterile 1 ml pipette tip held vertically, dragged from top towards the bottom of the well in a straight line with moderate speed and consistent pressure. Cells were washed with Dulbecco's Phosphate Buffered Saline (Sigma Aldrich, UK) to remove cellular debris and incubated in fresh medium in the absence or presence of the test sample. Cells were treated with estrogen (10^{-7} M), apigenin, luteolin, quercetin, kaempferol or chrysin at concentration of 30 µM, respectively. The concentration of phytoestrogens was selected as it falls within concentration range that results in significant effects in other phytoestrogen studies (6). After 24 hours incubation at 37 °C and 5% CO_2, cells were washed and visualized with crystal violet.

Animals and wounding

Animal studies were conducted at the University of Manchester in accordance with UK Home Office regulations using female C57BL/6 mice. Ten-week-old mice were assigned to the following groups; mice with intact ovaries (n = 5) and mice that had undergone ovariectomy (Ovx). Mice were wounded by making 1-cm-long horizontal incisions through the skin layer at 1 cm from the base of skull. One day prior to wounding, Ovx mice were injected subcutaneously at the wound site with apigenin (n = 6), luteolin (n = 6) or chrysin (n = 6) dissolved in 10% DMSO/90% corn oil with final concentration of 30 mg/ml (1.5 mg/mouse/day) or vehicle alone (n = 6). The dose of phytoestrogen used has previously been shown to be pharmacologically active in rodents (16). Two further injection were given on the day of wounding and one day after wounding. Estrogen-treated mice group was given with slow-release 17β-estradiol pellet (0.05 mg) (Innovative Research of America, Sarasota, FL, USA) at the time of wounding.

Histology and immunohistochemistry

At day 3 post-wounding, wound samples were collected and subjected to fixation in 10% buffered formalin followed by paraffin embedding. 6 µm sections were stained in either haematoxylin and eosin (H&E) or immunoperoxidase staining with rat anti-Mac-3 antibody (BD Biosciences Pharmingen, UK).

Image analysis

All images were taken using a Nikon Eclipse E600 microscope with a SPOT camera and software (Image Solutions Inc., Preston, UK). Images for scratch wound were taken at three different points along the scratch using 4x magnification. Images for wound measurements and macrophage quantification were taken at 4x and 20x magnification, respectively. All images were processed, analysed and compiled using Image Pro-Plus software (Media Cybernetics, Finchampstead, UK) and Adobe Photoshop.

Quantitation and data analysis

Scratch width, measured at five different points, was taken and calculated as percentage of wound closure as described below:

$$\% \text{ Wound closure} = \frac{\text{Scratch width at 0 hour} - \text{Scratch width after 24 hours}}{\text{Scratch width at 0 hour}} \times 100$$

Wound area was measured from the region below the clot until above the panniculus carnosus muscle and to the margins of normal skin on either side of the wound. Percentage re-epithelialisation was determined by dividing sum of lengths of newly formed epidermis from both wound edges by the total distance the epidermis would have to migrate between edges in order to close the wound. Quantification of macrophage density was performed using five different areas of wound granulation tissue and calculated for average macrophage density per mm^2 wound. Normality tests were carried out using Shapiro-Wilk normality test. If the dataset was normally distributed, one-way ANOVA was performed. For a non-parametric data, a Kruskal-Wallis test was performed. p

value of <0.05 was considered significant.

RESULTS

Phytoestrogens promote fibroblast migration *in vitro*

As a first step towards determining whether phytoestrogens could potentially promote wound closure, it is crucial to establish whether these compounds could affect the behaviour of a wound-relevant cell type and reproduce a known effect of estrogen. Fibroblasts play a number of key roles in wound closure and are recruited to wounds in large numbers. As a major wound cell type, fibroblasts present in most of wound healing phases. The migratory activity of fibroblasts bears important roles in initiating the proliferative phase and promotes wound contraction process that is desirable to reduce healing time. To examine whether phytoestrogens affect fibroblasts *in vitro*, in particular their ability to migrate, a scratch wound assay was performed. This simple assay measures the speed with which a scratch in a monolayer of cells is closed. Since cell migration is required for wound healing, this assay could provide initial evidence of phytoestrogens role in wound healing. Evaluation of the effect of phytoestrogens on fibroblasts migration were performed by treating a scratched fibroblast monolayer with 30 µM apigenin, luteolin, chrysin, quercetin and kaempferol. The 30 µM dose was selected because it falls within the concentration range used in other phytoestrogen studies that causes statistically significant effects when compared to estrogen (6). After 24 hours incubation at 37 °C with 5% CO_2, scratch width was compared at 0 hour to that at 24 hours to obtain percentage wound closure (Fig. 1A). It was expected that phytoestrogens treatment may alter fibroblast migration and thus affect the extent of wound closure. Estrogen treatment was found to lead to a significant increase in percentage of wound closure, from 47% for controls to 70% for estrogen treated (Fig. 1B). Treatment with

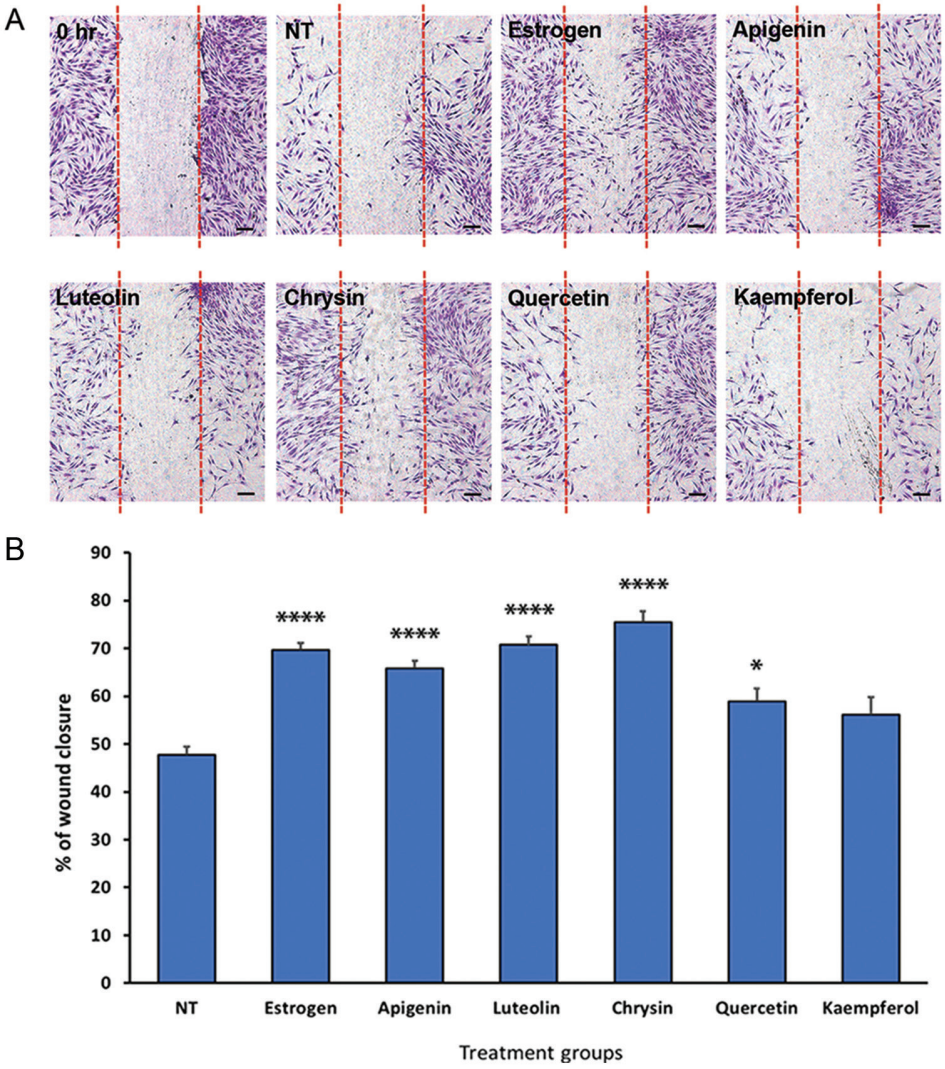

Figure 1: Phytoestrogens promote fibroblast migration in scratch wound assay. (A) Representative images of wound gap at 0 hour along with nontreated (NT) and treated fibroblasts after 24 hours incubation at 37 °C, 5% CO_2. Dotted lines refer to edge of the scratch. Scratch width was measured from right leading edge to left leading edge at five different points. (B) Graph showing percentage wound closure after 24 hours in which fibroblast migration is significantly promoted in all treatment except for kaempferol. Data represent mean ± SEM from three independent experiments. Bar, 200 µm. Statistical analysis was done using ANOVA test with Dunnett's correction for multiple comparisons. *, $p < 0.05$ and ****, $p < 0.0001$. All scale bars indicate 100 µm.

all phytoestrogen compounds resulted in an increased percentage wound closure relative to controls and this was statistically significant for all compounds except kaempferol. Chrysin had the strongest effect, increasing percentage wound closure 75%, a greater effect than estrogen. This is followed by luteolin, apigenin and quercetin which resulted in 71%, 67% and 60% wound closure, respectively. These results suggest that all the phytoestrogen compounds with the exception of kaempferol promote fibroblast migration *in vitro* to an extent comparable to estrogen.

Treatment with chrysin promotes skin wound healing in Ovx mice

Scratch wound assay finding that chrysin, luteolin, apigenin and quercetin can mimic the effect on estrogen in promoting fibroblast scratch wound healing *in vitro* suggested that these compounds could potentially also mimic the effect of estrogen in promoting wound closure *in vivo*. Therefore, it is important to investigate the effect of the three most potential phytoestrogens *in vivo* using the Ovx mouse model. Surgery to remove ovaries from the mice was performed in order to reduce the levels of endogenous estrogens and simulate age-induced impaired wound healing. The Ovx mice were then treated with 30 mg/ml chrysin, luteolin and apigenin for three consecutive days by subcutaneous injection, starting one day before wounding. 1 cm incisional wounds were made on the backs of mice. Wound tissues obtained at day 3 post wounding were fixed and stained (Fig. 2A). Upon imaging, the extent of healing was assessed by measuring the wound area and percentage of re-epithelialisation. As expected and consistent with previous studies (6), it was found that wounds

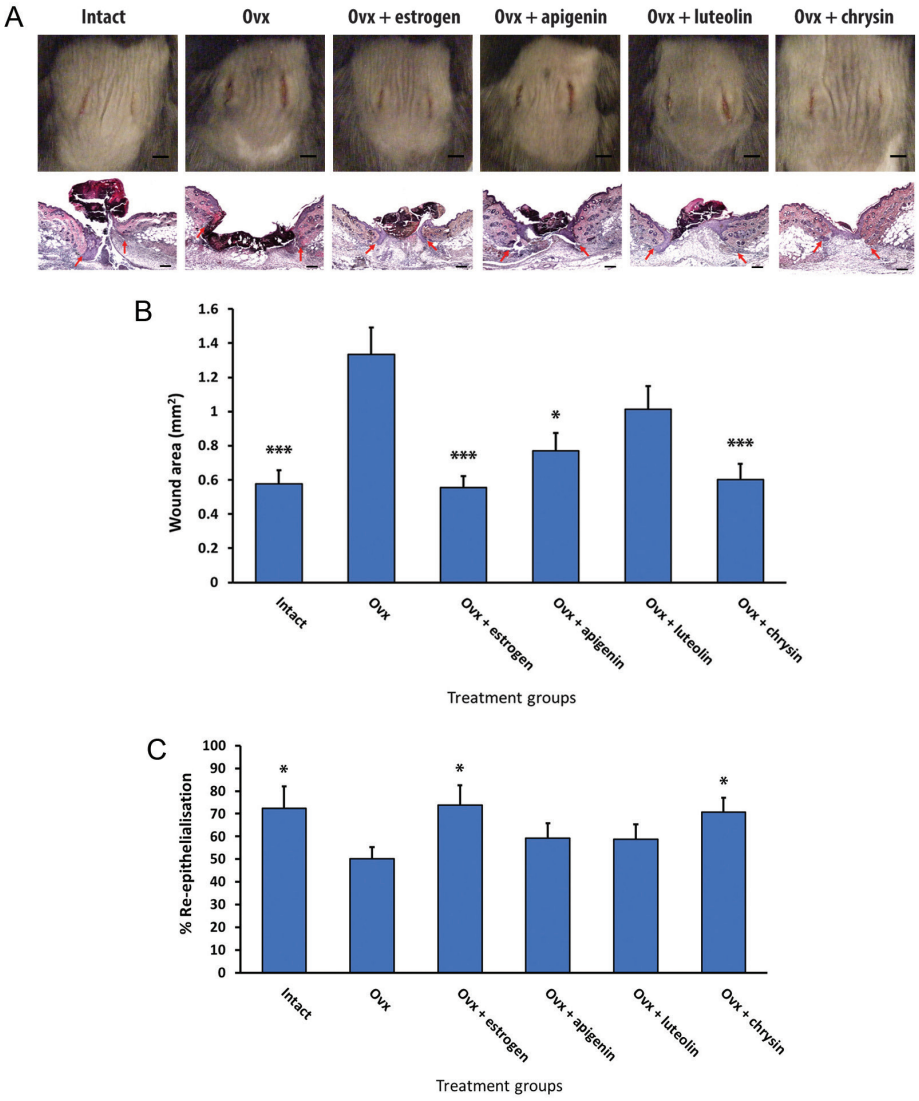

Figure 2: Chrysin, but not luteolin or apigenin, promotes wound healing. (A) Representative images of recovery wound at day 3 on mice dorsal (top panel) and H&E staining of the respective wound tissues (bottom panel). Red arrows indicate wound edges. Graphs showing (B) area of wound and (C) re-epithelialisation percentage of wounds in mice with intact ovaries, untreated Ovx mice and phytoestrogen-treated Ovx mice. The effects of chrysin in reducing the area of wound and elevating percentage of re-epithelialisation are comparable to those observed in mice with intact ovaries. n = 5 mice with intact ovaries, n = 6 Ovx mice untreated and n = 6 Ovx mice per treatment. Data represent mean ± SEM. Statistical analysis was done using ANOVA test with Dunnett's correction for multiple comparisons. *, $p < 0.05$ and ***, $p < 0.001$. All scale bars indicate 200 µm.

in mice with intact ovaries exhibited a significantly decreased wound area and increased percentage of re-epithelialisation compared to vehicle treated Ovx mice (Fig. 2B and 2C), with wound area at 3 days post wound increased from 0.58 mm^2 in controls to 1.33 mm^2 in Ovx mice and percentage re-epithelialisation decreased from 72% to 50%. Treatment with chrysin resulted in a massive reduction of wound area and increased re-epithelialisation percentage compared to vehicle treated Ovx mice, with area reduced to 0.60 mm^2 and percentage re-epithelialisation increased to 71%. These wound measurements were comparable and not significantly different to those observed for Ovx mice treated with estrogen. Apigenin also resulted in significantly reduced wound area compared to controls, although the reduction was less than observed for chrysin. Percentage re-epithelialisation was not significantly different for this compound. Luteolin treatment resulted in reduced wound size and increased re-epithelialisation compared to controls, however these differences were not statistically significant. These results suggest that phytoestrogens, in particular chrysin, may be potent promoters of wound healing in individuals with reduced estrogen levels.

Macrophage infiltration is reduced in chrysin-treated wound

Estrogen has been suggested to exert anti-inflammatory effects during wound healing whereby its absence in mice leads to increase macrophage infiltration in the wound tissues (17). Since it was observed that skin wound healing in Ovx mice was accelerated when treated with phytoestrogens, it would be interested to know whether phytoestrogen treatment also affects the inflammatory response in the wound tissues. To do this, the wound tissues were fixed and stained with the Mac-3 antibody, which recognized macrophages (Fig. 3A). Number of macrophages was quantified in five different areas in the wound granulation tissue and average macrophage density per mm^2 wound was calculated. Macrophage density in wounds of mice with intact ovaries was found significantly reduced to 272 macrophages/mm^2 wound compared to vehicle treated Ovx mice (848 macrophages/mm^2 wound) (Fig. 3B). Treatment with chrysin resulted in a similar effect when compared to vehicle treated Ovx mice, with a significant reduction in macrophage density to 293 macrophages per wound area. The effect of chrysin on macrophage density was also found to be comparable

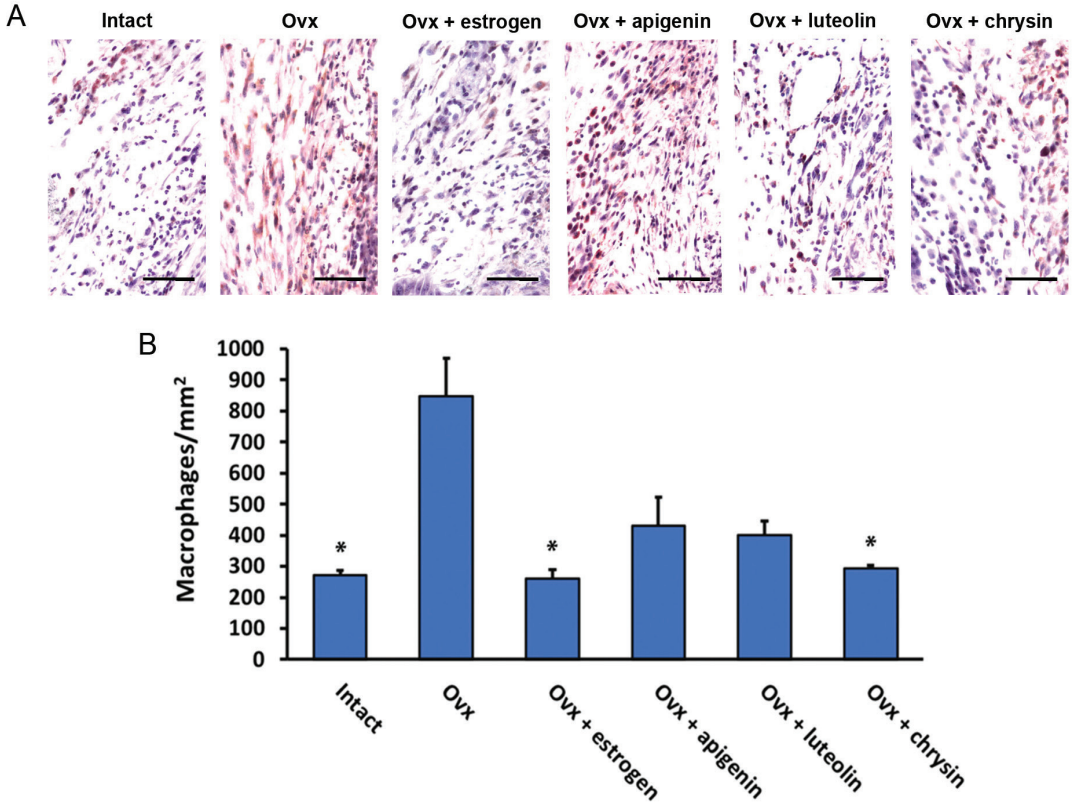

Figure 3: Chrysin reduces macrophage infiltration in wound granulation tissues in Ovx mice. (A) Representative images of macrophage staining from wound tissues at day 3 of mice with intact ovaries along with untreated and treated Ovx mice. (B) Graph showing macrophages density per wound area (mm^2) with a significant reduction in both mice with intact ovaries and chrysin-treated Ovx mice. n = 5 mice with intact ovaries, n = 6 Ovx mice untreated and n = 6 Ovx mice per treatment. Data represent mean values ± SEM. Statistical analysis was done using ANOVA test with Dunnett's correction for multiple comparisons. *, $p < 0.05$. All scale bars indicate 40 µm.

with that observed for mice treated with estrogen. Both luteolin and apigenin treatment resulted in a decrease macrophage density; however, the differences were not statistically significant. Collectively, these findings are consistent with those observed in wound measurements (wound area and percentage re-epithelialisation) and suggest that a reduced inflammatory response may contribute to improved wound healing in chrysin treated Ovx mice.

DISCUSSION

Findings from the fibroblast scratch wound assay demonstrated the potential of four phytoestrogens; chrysin, luteolin, apigenin and quercetin to trigger migration of fibroblasts to a similar extent to estrogen. Estrogen has previously been reported to promote migration of several types of cells (6,18,19). The mechanism of action of estrogen has been studied by pharmacological activation of estrogen receptor (ER) isoform in mice and this showed that estrogen promotion of fibroblast migration is mediated by ERβ signalling activation, while ERβ does not have significant role in this process (17). This indicates a receptor-specific signalling effects in fibroblasts migration. Based on immunohistochemical studies, ERβ was found to be expressed at a higher level in human skin compared to ERβ (20). This ubiquitous expression suggests that ERβ but not ERβ plays a significant role in mediating estrogenic effect in human skin (21). It is currently unknown whether the stimulatory effects of chrysin, luteolin, apigenin and quercetin are mediated via ERβor a different signalling pathway. Hence, a more detail investigation on the mechanism is required.

The scratch wound assay performed in the present study is a basic technique for investigating cell migration in which a vertical wound is created through the cell monolayer by scraping using a pipette tip. The major advantages of this *in vitro* experiment are inexpensive because it does not require special equipment or expensive reagents, faster method and relatively easy to handle. The wound healing assay can be considered for fast screening of potential therapeutic anti-cancer drugs for their migratory function. However, main drawback of this approach is that the wound area produced by scratching can be irregular and forms crooked leading edges, which eventually contributes to variable results between experiments. This problem could be due to poor scratching technique especially when scraping is done slowly and bent. To overcome this limitation, the pipette tip should be held vertically and dragged through the cell monolayer with modest speed and even pressure. Alternatively, miniature razor blades or commercial tools are recommended to produce high quality wounds rather than using a pipette tip (22). Another limitation of scratch wound assay is the difficulty to distinguish the exact wound sites at a later time point owing to the fact that the boundaries of the scratch are ragged and the cells at the scratch edges often migrate into the wound gap at different rates. Taking multiple measurements of the scratch width at various positions may reduce the variability by generating an average percentage of wound closure. Ideally, live-cell imaging will enable researcher to capture images in identical locations at multiple time points without subjective error (23).

Binding affinities of apigenin and kaempferol for ERβ were found to be 2- to 5.5-fold higher than for ERβ, while binding affinities ratio for luteolin, chrysin and quercetin were not determined (24). In a different study, quercetin has been suggested to potentially elevate the stimulation of ERβ as it activates ERβ to a 4.5-fold higher than estrogen and to a lesser extent in ERβ (25). The degree of ER binding affinity has been suggested to be influenced by the structural properties of the compounds. Kaempferol has a hydroxyl group in the 3-position and it is suggested that a hydroxyl group substitution at the C-3 position in ring C enhances binding specificity. Strong binding specificity for estrogen receptors however does not seem to significantly influence the migration activity of kaempferol-treated dermal fibroblasts in the present study. Treatment with quercetin showed normal fibroblasts migration while other studies have reported delayed wound closure in quercetin-treated cells plated on an artificial extracellular matrix due to the ability of quercetin to alter integrins expression (26). Both quercetin and kaempferol are classified under the same subclass, flavonol, due to their structural backbone similarities. Flavonols are distinguished from the flavone subclass by having an extra hydroxyl group at the C-3 position.

Flavonols are also reported to exert strong inhibitory activity on matrix metalloproteinases (MMPs) (27). MMPs have been demonstrated to play a crucial role in wound repair, mainly by modifying the extracellular matrix microenvironment to allow cell adhesion and migration for tissue reformation (28). Kaempferol and quercetin have been found to down regulate the expression of MMP-1 in dermal fibroblast through inhibitory activity of the transcription factor, activator protein-1 (AP-1) (29,30). This might explain the relatively weak effect of kaempferol and quercetin in the present study. By contrast, apigenin, luteolin and chrysin all act as weak collagenase inhibitors in dermal fibroblasts (29,31). Having this characteristic seems to be associated with promoting fibroblast motility as observed in the scratch wound assay. The same effect has been observed for another phytoestrogen, genistein, which accelerates migration of dermal fibroblast *in vitro* (6), consistent with lack of ability of genistein in suppressing MMP-1 (29).

Healing promoting potential of apigenin, luteolin and chrysin was studied in the well-characterised murine model, Ovx mice, to investigate whether these flavones can compensate estrogen depletion in human age-

related delayed wound healing. Complementing the *in vitro* data, it is interesting that of the flavones, only chrysin was found to exhibit significant accelerating effects on wound repair *in vivo*. Treatment with luteolin and apigenin resulted in a trend towards improved wound healing compared to Ovx mice, but this was not statistically significant in this study. Future work should be carried out on dissecting the underlying mechanisms of epidermal regeneration by chrysin which may involve the use of epidermal differentiation markers such as K10, K14, filaggrin, DSG-1 and CDSN.

Reduced macrophages infiltration in chrysin-treated Ovx mice in the present study suggests a potential mechanism by which chrysin influences M1/M2 polarization of macrophage (32). Chrysin triggers a transition from pro-inflammatory M1 to anti-inflammatory M2 phenotype by activating the peroxisome proliferator activated receptor γ (PPARγ) and also target genes of PPARγ such as the CD36 and Arg1. PPARγ attenuates inflammation through the inhibition of many proinflammatory genes such as interleukin (IL)-1, IL-6, tumour necrosis factor (TNF)-α and inducible nitric oxide synthase (iNOS). As the Ovx mouse wound was used to represent the chronic wound occurs in the elderly, which could be due to prolonged inflammation, the time point of day 3 after wounding was presented. Investigation at later time points (day 7 and day 10 after wounding) should be carried out in the near future to evaluate the potential of phytoestrogens in accelerating the proliferative and remodelling phase in Ovx mice model.

Other studies have revealed a cross talk between ER signalling and Insulin-Like Growth Factor-1 (IGF-1) signalling in regulating cell migratory activity (33). Intriguingly, it has been found that introduction of IGF-1 in estrogen-depleted mice can promote healing of wounds to the same extent as estrogen action via ERβ signalling. The wound healing effects of other phytoestrogen, genistein, has been suggested to be mediated via IGF-1 receptor (6), hence further investigation is required to unravel the mechanism of action of chrysin in wound healing. While estrogen has been implicated in mouse anti-inflammatory response during wound healing mediating through the ERβ receptor (34), it seems beneficial to perform an experiment using estrogen responsive element (ERE)-luciferase reporter mice (35) to determine whether the effects observed in the Ovx model are mediated via ER-dependent or ER-independent pathways. In addition, co-treatment with ER antagonists would allow further examination whether there is an ER antagonism effect on the ability of chrysin to hasten the healing process. It is possible that other biological properties of chrysin for example, in preventing free radicals and microbial invasion, might also contribute to the healing effects (36,37).

CONCLUSION

The present study demonstrated that among a group of naturally-ocurring estrogen analogues, the effects of chrysin on wound healing may be at least partly due to its ability to influence fibroblast migration and macrophage recruitment, however it is possible that effects on other cell types are also important. These findings could serve as evidence that chrysin could potentially be used as a therapy for impaired wound healing in the aged.

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