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Original Article

Young coconut juice accelerates cutaneous wound healing by downregulating macrophage migration inhibitory factor (MIF) in ovariectomized rats: Preliminary novel findings

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Abstract

Estrogens play a crucial role in cutaneous wound healing by down-regulating macrophage migration inhibitory factor (MIF). We had previously reported the effect of young coconut juice (YCJ) known to contain the phytoestrogen, β-sitosterol, on cutaneous wound healing in ovariectomized (ovx) rats. This research investigated the possible mechanisms of YCJ on cutaneous wound healing and it was found that it down regulated macrophage migration inhibitory factor (MIF). This resulted in ultrastructural changes that were observed using transmission electron microscopy (TEM). Four groups of female rats (6 in each group) were included in this study: sham-operated, ovariectomized (ovx), ovx that received estradiol benzoate (EB) injections intraperitoneally, and ovx that received YCJ orally. Two weeks after ovariectomy, two equidistant 1-cm full-thickness skin incisional wounds were made. At the end of the third week (7 day treatment) and the fourth week (14 day treatment) of study the rats were sacrificed, and their serum estradiol (E2) levels were measured by a chemiluminescent immunoassay. The skin from the wound was excised and examined by TEM and MIF immunohistochemical staining. The TEM study after 14 days of treatment showed that the size of the keratinocyte cells from the ovx+YCJ group was larger and these cells contained many more cytoplasmic processes than those of the ovx group. The MIF immunoreactivity was also lowest in the ovx+YCJ group. This study showed that there was an increased intercellular exchange via the cytoplasmic processes of the keratinocytes that could account for the promotion of cutaneous wound healing in the ovx rats receiving YCJ, and that the possible mechanism for this was via the down-regulation of MIF.

Keywords: young-coconut juice, *Cocos nucifera* L., macrophage migration inhibitory factor (MIF), ovariectomy, transmission electron microscopy (TEM)

1. Introduction

The increase in the numbers of the elderly has produced a parallel increase in morbidity associated with age-related delayed wound healing, and resulted in an increase of costs to the health services. Estrogens play a crucial role in cutaneous wound healing and repair, which is significantly delayed in their absence (Ashcroft *et al.,* 1999, 2003). Exogenous estrogen (17β-estradiol) can reverse the delay in wound healing, reduce inflammation and stimulate re-epithelialisation (Ashcroft *et al.,* 1997, 1999).

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In postmenopausal women, a decline of estrogen levels is associated with a variety of cutaneous changes and impaired wound healing. In addition, phytoestrogens such as daidezein, genistein, isoflavones in soybeans, reversterol in grapes and red wine can bind both ERα and ERβ, and have positive effects on human skin e.g. by reducing UV-induced cell death of cultured keratinocytes, improving skin elasticity, reducing wrinkle depth and increasing the production of type I procollagen (see reviews in Thornton, 2013). We previously found that YCJ containing an estrogen-like hormone, β-sitosterol, has an effect on accelerating wound healing in ovx rats, a model often used for postmenopausal women (Radenahmad *et al.,* 2006, 2012). The pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) is a master regulator of the effects of estrogens on wound healing and the *in vitro* MIF production was down-regulated by 17b-estradiol (Hardman *et al.,* 2005; Shimizu, 2005). Using RT-PCR and Southern blot techniques, Abe and colleagues found that expression of MIF mRNA during wound healing of rat skin increased from 1 to 24 hours after injury. Immunochemical analysis also confirmed the enhanced expression of MIF in the wounded tissue (Abe *et al.,* 2000). With this background, YCJ was further investigated for its possible mechanism on accelerating wound healing in ovx rats, using anti-MIF antibody. In addition, we found that in the ovx+ YCJ group, not only was the wound healing accelerated, but also the epidermis and dermis were thicker than those in the control groups (sham, ovx and ovx+EB groups). The epidermis is a stratified squamous epithelium comprising predominately keratinocytes, that can be divided into basale, spinosum, granulosum and corneum cells on the basis of their morphologies. Therefore, in the present study, keratinocyte morphological changes, by observation only, at the ultrastructural (TEM) level were further investigated.

2. MAterials and Methods

2.1 Plant material

Young coconut juice (*Cocos nucifera* L., Arecaceae) was collected from the Tungngai district, Hat Yai, Songkhla, Thailand. It was authenticated by Associate Professor Dr. Sanan Subhadhirasakul at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Thailand. YCJ was freeze dried, and the powdered form was kept at -30°C until used. This powder was freshly reconstituted and prepared for oral intake every day. A complete description of YCJ, including its preparation and administration, has been provided in previous publications (Radenahmad *et al.,* 2009, 2011).

2.2 Animals

All animals used were adult two-month old female Wistar rats weighing approximately 230 g. The animals were housed in a controlled environment at 25±1°C on an illumination schedule of 12h light/12h dark cycle. Rats had unrestricted access to standard pellet food and water. The study was approved by the Committee on Animal Care and carried out in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Prince of Songkla University.

2.3 Experimental design

There were four animal groups (6 rats per group) included in this study. The first group consisted of ovx rats, the second group were sham-operated rats, the third group were ovx rats injected intraperitoneally with exogenous estrogen (2.5mg/kgBW of estradiol benzoate, EB) once a day every day, and the fourth group included ovx rats that received YCJ (100mL/kgBW/day). The dose of EB and YCJ in this study was based on that reported in our earlier studies and in which dose standardization and optimal administration were established (Radenahmad *et al.,* 2006, 2009, 2011). In this study, the administration of EB and YCJ was started two weeks after performing overiectomy. Rats belonging to the first and second groups received deionizied water instead of EB and YCJ. Two weeks after sham-operation or ovariectomy, all animals were wounded by making an excision at the dorsal surface 1 cm below the scapula, 1.5 cm long and 3 mm deep (from the skin and panniculus carnosus muscle). The incision was left to heal by secondary intention (i.e. the wound edges were not closed by sutures). The rats were force fed with deionized water, YCJ or given an injection of EB for another 7 (called 7-day treatment) or 14 days (called 14-day treatment). At the end of the experimentation, animals were sacrificed, the wound sections were bisected and processed so that the midpoint of the wound was sectioned and could be compared between groups. Original wounded tissues were harvested at days 8 and 15 after wounding and were disected for routine H&E, transmission electron microscopy (TEM) and immunohistochemical staining. Serum was collected for estradiol measurements using chemiluminescent immuno-assays (CIA).

2.4 Immunohistochemistry

Tissue samples from the dorsal skin were processed for paraffin blocks, and six 5μm sections were collected from each block and mounted on 3-aminopropyltriethoxysilane (TESPA; Sigma)-coated slides. The first two sections were stained with H&E, and were examined by light microscopy for anatomical orientation. The remaining three sections were immunostained with the rabbit anti-rat migration inhibitory factor (MIF) monoclonal antibody (M3895-07G United States Biological, P.O. Box 261, Swampscott, Massachusetts 01907), while the remaining one section was used as a negative control. The immunostaining technique was described previously (Radenahmad *et al.,* 2009). The primary antibody used was anti-MIF, at a dilution of 1:1,000. At the final step,

sections were counterstained with Meyer's hematoxilin for five seconds, and cover-slipped. Negative controls included sections in which incubation with the primary antibody was replaced by Tris buffer solution (TBS). No staining was seen in any of these control sections.

2.5 Microscopic analysis by quantitative histomorphometry

MIF-immunoreactive (-ir) scoring was analyzed by two independent observers. Readings from both observers were then added and the average was determined. The score was analyzed by using image analysis and semi-quantification was performed using an Image Pro Plus program (DP11, Olympus SZX 12, Japan). The mean values ± SEM were used to compare the data of the four groups.

MIF immunostaining scoring:

0 = the least number of MIF-ir hair follicles

4 = the highest number of MIF-ir hair follicles

Number of MIF-ir hair follicles/field	Scores
0 follicle/field	0
1-3 follicles/field	1
4-6 follicles/field	2
7-9 follicles/field	3
≥ 10 follicles/field	4

2.6 Specimen Preparation for TEM

Tissue samples from the dorsal skin of the original wounds were first fixed in 4% paraformaldehyde (primary fixation) for 24 hours at 4°C and then in 1% OsO_4 for 1 hour, washed with phosphate buffer solution and emblocked with 2% uranyl acetate for 1 hour. Dehydration was through ascending concentrations of ethanol and specimens were then embedded in Embed-812 (Epon-812 Substitute, Cat # 14900). Semithin sections were cut at 400 nm and stained with 0.5% toluidine blue for checking the tissue area. Ultrathin sections were cut at 90 nm and stained with 5% uranyl acetate and lead citrate before being observed using a transmission electron microscope (JEOL JEM-2010, Japan). Micrographs were taken at a magnification of 6000x.

2.7 Serum estradiol

All the rats were sacrificed on the first day of the fourth and the fifth week, and their serum was collected and measured for estradiol (E2) using the chemiluminescent immunoassay (CIA) technique (ECLIA, Modular E 170C, Estradiol II 03000079 122, Roche, Germany).

2.8 Statistical analysis

The data was analyzed by calculating a mean and the standard error of the mean (SEM). Statistical analysis was performed using the Kruskal-Wallis and the Mann-Whitney U-tests. Data were considered statistically significant at the $p<0.05$ level.

3. Results

3.1 MIF immunohistochemistry

MIF immunoreactivity was detected in all skin layers: epidermis, dermis, hypodermis and also at the hair follicles,

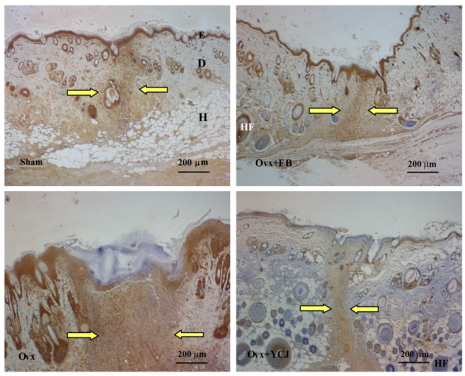

Figure 1. Expression of MIF immunoreactivity staining in female rat skins after 14 days of treatment. Yellow arrows indicate the wound areas. E = epidermis, D = dermis, H = hypodermis, HF = hair follicle. Ovx = ovariectomized group; Ovx + EB = ovariectomized group receiving estradiol benzoate; Ovx + YCJ = ovariectomized group receiving young coconut juice. Each group consisted of 6 rats.

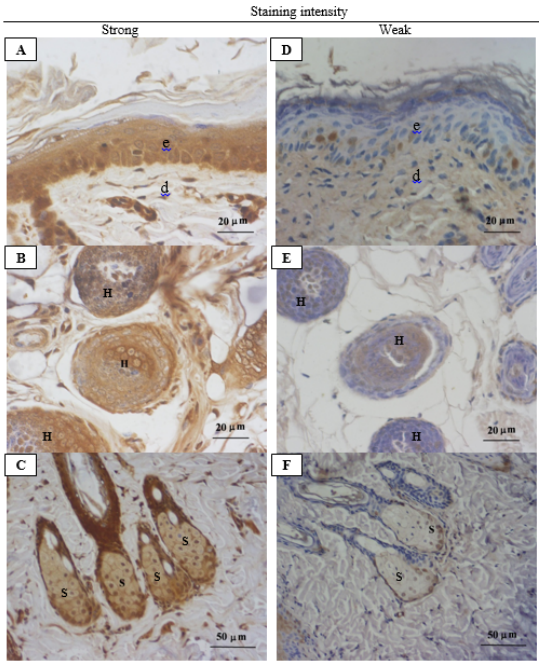

Figure 2. Varieties of MIF immunoreactivity in rat skin: left column (A-C) represents strong MIF immunoreactivity; right column (D-F) represents weak MIF immunoreactivity. e = epithelium, d = dermis, H = hair follicle, S = sebaceous gland.

sebaceous glands, fat cells, skeletal muscles and fibroblasts (Figures 1 and 2). Compared to the sham, ovx+EB and ovx groups, the MIF immunoreactivity of the ovx+YCJ group was the lowest (Figures 3 and 4A). The immunoreactivity of the ovx+YCJ group was close to the negative (-ve) control group (Figure 3). The MIF immunostaining scores in the ovx+YCJ group after both 7 days and 14 days of treatment were lower compared with the ovx group at $p<0.05$ and $p<0.01$ respectively (Figure 4A).

After 7 days of treatment, when the serum E2 levels were statistically correlated with the MIF immunostaining scores, the p-value of testing regression equation: $y = 0.0059x + 1.6797$ is 0.317 leading to the conclusion that serum E2 level of 7 day treatment increased MIF-ir scores. In contrast, in 14 days treatment, the p-value of testing regression equation: $y = -0.0212x + 3.3746$ is 0.00095 leading to the conclusion that serum E2 level of 14 day treatment reduced MIF-ir scores (Figure 4B).

3.2 TEM

After 7 days of treatment, the size of the keratinocytes of the epidermis from all groups was almost the same (Figure 5). After 14 days of treatment, the TEM micrographs of the keratinocyte of the epidermis showed that the cell size of the ovx and ovx+EB groups was smallest and the size was restored in the ovx+YCJ group (Figure 6). The size of the keratinocytes of the ovx+YCJ group was even larger than that of the ovx+EB group. TEM examination demonstrated that the epidermal keratinocytes were connected tightly through desmosomes in the sham, ovx+EB and ovx+YCJ groups. Desmosomes were found in the ovx epidermis but they were fewer and much smaller than those of the control groups (sham and ovx+EB groups) and ovx+YCJ group (Figure 6). The cytoplasmic processes of the keratinocytes of the ovx+YCJ group were largest and the numbers also

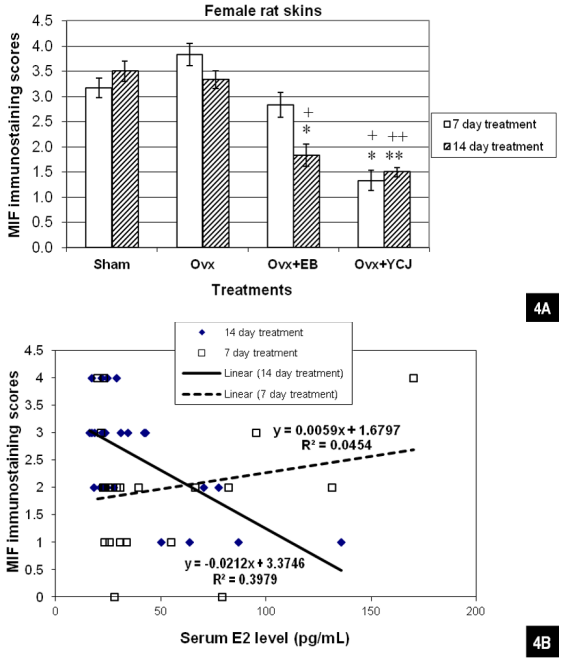

Figure 4. A) Expression of MIF intensity (mean ± SEM) in the female rat skins. The MIF intensity of the ovx+YCJ group was markedly decreased after 7 and 14 days of treatment compared with the ovx group. The MIF intensity of the ovx+EB was significantly decreased only after 14 days of treatment compared with the ovx group. Ovx = ovariectomized group; Ovx + EB = ovariectomized group receiving estradiol benzoate; Ovx + YCJ = ovariectomized group receiving young coconut juice. Each group consisted of 6 rats. Data is expressed as a mean ± SEM. *, ** $p<0.05$, $p<0.01$ respectively compared with sham group. +, ++ $p< 0.05$, $p<0.01$ respectively compared with ovx group. B) Regression correlations between the MIF intensity and the serum E2 levels of the four groups examined. R^2 = -0.40 after 14 days treatment and R^2 = 0.05 after 7 days treatment.

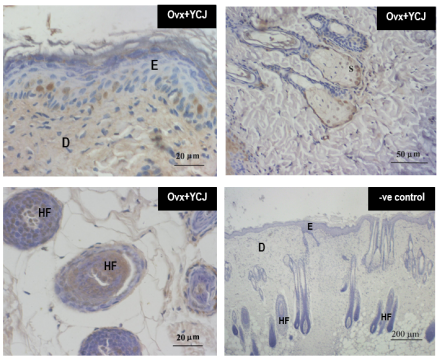

Figure 3. MIF immunoreactivity in rat skin after 14 days treatment. MIF immunoreactivity in the ovx+YCJ group was obviously close to the negative control (-ve control). E = epidermis, D = dermis, S = sebaceous gland, HF = hair follicle.

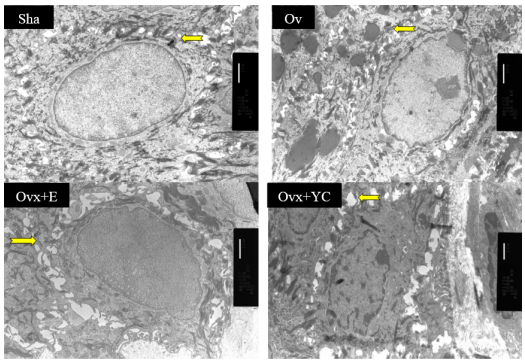

Figure 5. TEM micrograph of keratinocyte cells of the epidermis after 7 days treatment (6000x). The yellow arrows indicate the cytoplasmic processes of the keratinocyte cells. Ovx = ovariectomized group; Ovx + EB = ovariectomized group receiving estradiol benzoate; Ovx + YCJ = ovariectomized group receiving young coconut juice. Bar = 1 µm.

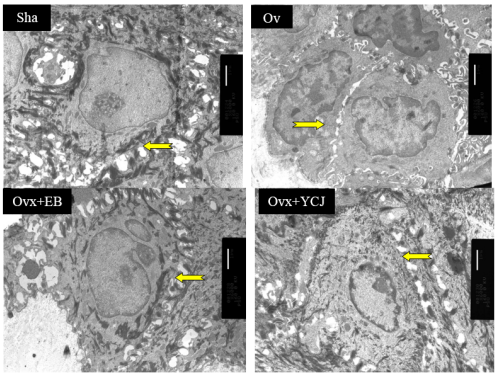

Figure 6. TEM micrograph of the keratinocytes of the epidermis after 14 days treatment (6000x). The yellow arrows indicate the cytoplasmic processes of the keratinocyte cells. Note the shrinkage of the size of the keratinocyte cells of the ovx group. Cytoplasmic processes of ovx+YCJ group were larger than that of ovx group. Ovx = ovariectomized group; Ovx + EB = ovariectomized group receiving estradiol benzoate; Ovx + YCJ = ovariectomized group receiving young coconut juice. Bar = 1 μm.

increased, as based on the observations of an overall histological evaluation (Figure 6)

4. Discussion

Tissue repair mechanisms are complex. They involve inflammation, granulation and remodelling of injured tissues. Several cytokines or growth factors play important roles during tissue repair and promote wound healing (Sporn and Roberts, 1986). Shimizu found that keratinocytes migrated from the wound edges and their proliferation was essential for proper dermal wound repair (Shimizu *et al.,* 1999). For the overall wound healing process, many cytokines and inflammatory mediators released from the keratinocytes influence events by regulating the growth, differentiation and metabolism of the other cells that are associated with the injured skin lesion (Sporn and Roberts, 1986). For example, an enhanced MIF mRNA expression was detected in wounded skin, and a higher amount of secreted MIF was produced by fibroblasts cultured from wounded skin when compared with normal skin fibroblasts (Abe *et al.,* 2000). Anti-MIF antibodies also induced a delay in wound healing *in vivo* (Shimizu, 2005). Altogether, these results indicated that MIF plays an important role in would healing e.g. it serves as a multifunctional cytokine, not only during inflammation and immune responses, but also in cell proliferation and angiogenesis during the wound healing processes (see reviews in Shimizu, 2005).

Hardman *et al.,* (2005) and Shimizu (2005) found that MIF, the pro-inflammatory cytokine, was the major regulator for the effects of estrogens on wound healing and that, *in vitro,* MIF production was down regulated by 17β-estradiol. In this study, our results using the anti-MIF antibody and immunohistochemical techniques have added support to the results from those experiments. We also, showed slightly different results in some aspects. For example, in the present study, MIF immunoreactivity was detected in all layers of the skin: epidermis, dermis and hypodermis, which is in contrast to the results of Ashcroft *et al.* (2003), who found that MIF immunoreactivity was detected only in the suprabasal layers in the migrating epidermis. These differences might arise from the different rodents used in the experiments as well as the period the animals were left after ovx before testing. In our experiments we used Wistar rats and the rats were left for 2 weeks after ovx before making the excisional wounds while Ashcroft *et al.* (2003) used mice and ovx was carried out 1 month before commencing the wounds. Due to the longer period of 1 month after ovx, the latter might need a longer time for keratinocytes to proliferate and to progress from the basal layer of the epidermis to the upper suprabasal layers. After 14 days of treatment, the MIF immunoreactive scores of the ovx+EB group was significantly lower than that of ovx group (Figure 4A). This agrees with the results of Ashcroft *et al.* (2003) who found that estrogen replacement in wild-type ovx mice resulted in a marked reduction in MIF levels and that process was involved with estrogen receptors.

Estrogen obviously binds to an estrogen receptor that results in a reduction of MIF expression (Ashcroft and Ashworth, 2003). Since estradiol (E2)-induced suppression of MIF transcription that was dependent on ERα (Ashcroft *et al.,* 1999; Hardman *et al.,* 2005) and our previous work clearly showed that accelerated wound healing was highly correlated with the ERα and ERβ of the hair follicles (Radenahmad *et al.,* 2012). Circulating E2 level was significantly ($p<0.01$) less in the ovx+YCJ group following 7 and 14 days intake, as compared to the other groups (Radenahmad *et al.,* 2012). In addition, the MIF immunoreactivity was lowest in the ovx+YCJ group compared with the sham, ovx+EB and ovx groups. This meant that YCJ containing the phytoestrogen, β-sitosterol, was a more potent repressor of MIF than exogenous estrogen (EB). Our results have been supported by Hardman's work who found that despite clearly different functions in some tissues e.g. the uterus, selective estrogen receptor modulators (SERMs), such as tamoxifen and raloxifene acted as estrogen agonists and potently accelerated cutaneous wound healing by decreasing MIF expression and that β-sitosterol was effectively better than estrogen at an equal molar concentration (Hardman *et al.,* 2008).

Another example that showed that phytoestrogen promoted wound healing was reported by Emmerson *et al.* (2010). They clearly demonstrated the beneficial effects of genistein on skin repair via multiple independent mechanisms. Genistein was found to reduce the number of wound cells that were expressing the MIF protein and the relative MIF expression (*in vitro*) was down regulated more in the genistein treated mice compared with the untreated ones (Emmerson *et al.,* 2010). Most of the phytoestrogens with wound healing properties are the flavonoids (see review in Havsteen, 2002). Recently, our team used gas chromatography-mass spectrometry and HPLC to confirm that the

phytoestrogens present in YCJ were sitosterol, stigmasterol, campesterol (Rujiralai and Sitaruno, 2009) and β-sitosterol (Ratanaburee *et al.* 2014). In this study we have demonstrated the beneficial effects of these phytosterols on cutaneous wound healing as the result of down regulating MIF expression *in vivo*.

Our previous results demonstrated that in the first week after wounding in the ovx+YCJ treated rats, there was little evidence for significant microscopic healing changes to the wound area, compared to the controls. However, after 2 weeks there were clear microscopic changes of accelerated wound healing compared to the controls such as a significant reduction of wound depth and width, an increased thickness of the epidermis and dermis, thicker and more abundant hair follicles, and the density of the immunostaining against ERα, and ERβ in the epidermis, dermis, and hypodermis (Radenahmad *et al.,* 2012). Hair follicles are predominantly composed of keratinoctyes and the importance of hair follicles in skin biology does not rest solely on their ability to produce hair. Hair follicles are self-renewing, and contain reservoirs of multipotent keratinocyte stem cells that are capable of regenerating the epidermis, and, accordingly, are thought to be actively involved in accelerating wound healing (Millar 2002; Kierszenbaum 2007). Our previous study supported this theory (Radenahmad *et al.,* 2012). This was also confirmed by a significant regression correlation and a TEM study in this present study. The longer the ovx rats were force fed with YCJ (14 days treatment), the lower was the immunostaining score for MIF compared to the 7 day treatment (Figure 4B). TEM micrographs of the keratinocyte of the epidermis showed that the cell size of the ovx and ovx +EB groups was smallest and the size was restored in the ovx+YCJ group (Figure 6). The size of the keratinocytes of the ovx+YCJ group was even larger than that of the ovx+EB group. TEM demonstrated that epidermal keratinocytes were connected tightly through desmosomes in the sham, ovx+EB and ovx+YCJ groups. Such desmosomes were found in the ovx epidermis but were fewer and much smaller than those of the control groups (sham and ovx+EB groups) and the ovx+YCJ group (Figures 5 and 6). The cytoplasmic processes of the keratinocytes of the ovx+YCJ group were the largest and their numbers were also increased (Figures 5 and 6). This was supported by other studies e.g. Bellemare *et al.* (2005) who showed that hypertrophic keratinocytes induced a significantly thicker dermis or the formation of more extracellular matrix than normal keratinocytes. They also demonstrated that keratinocytes had a role in influencing the proliferation of dermal cells and the accumulation of the matrix (Bellemare *et al.*, 2005). Therefore, enlarged cytoplasmic processes might help to increase the transfer of growth factors, cytokines etc. and this influenced the intercellular exchanges between adjacent keratinocytes and eventually accelerated wound healing.

Keratinocytes progress from the basal proliferating layer of the epidermis to the area immediately adjacent to the differentiating "spinous" layer and undergo significant structural changes such as the establishment of close intercellular interactions, formation of desmosomes and rearrangement of the actin/cytokeratin network. In the present study, desmosomes were present in greater numbers and had larger sizes in the ovx+YCJ groups when compared with other groups. The longer the ovx rats were treated with YCJ (14 days), the greater were the numbers of desmosomes (compared with the 7 day treatment) (Figures 5 and 6). This is in agreement with Calautti's work who used geneistein to test keratinocyte cell cultures from newborn Sencar mice. They found that the protrusions from the neighboring cells in the Genistein-treated cultures were more consistently observed compared with the untreated ones (Calautti *et al.,* 1998).

Compared to other skin rejuvenating agents such as AHAs (alpha hydroxyl acids), a class of compounds derived from food sources, YCJ has, so far, shown many benefits and no harmful effects such as those that occurred after the application of AHAs (Inan *et al.,* 2006). Prolonged consumption of YCJ is, however, being investigated to determine if there are any harmful side effects of drinking YCJ as a long-life beverage. The optimal dose of YCJ would then be adjusted to balance between any beneficial effects and side effects that might be observed.

5. Conclusions

In summary, we have demonstrated the clear beneficial effects of YCJ on cutaneuous wound healing as a consequence of down-regulating the macrophage migration inhibitory factor (MIF) in an ovariectomized rat model. The results from the present study were also confirmed by ultrastructural investigation using TEM. These encouraging findings could have exciting clinical implications in the future. Doses could be modified to have both therapeutic and cosmetic benefits such as accelerating wound healing and reducing the scars in surgery, particularly those associated with plastic surgery when the scars need to be minimized as much as possible after an operation; treating chronic ulcers; skin whitening; and repairing burns.

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