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The effect of isoflavone-daidzein oral medication on cutaneous wound healing in female ovariectomized mice

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Short title: Effect of daidzein on cutaneous wound healing

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Abstract

This study investigated the influence of oral administration of isoflavone-daidzein on the cutaneous wound healing process in female ovariectomized mice. Eight-week-old female mice were divided into groups of ovariectomized mice and mice administered daidzein after an ovariectomy. Two full-thickness wounds on the dorsum were made in mice in both groups. There was no significant difference between wound areas of the two groups from wounding to healing during 15 days. The area in the group administered daidzein tended to be smaller than that in the ovariectomized group during the inflammatory phase 4 and 5 days after wounding . The rate of re-epithelialization in the group administered daidzein tended to be higher than that in the ovariectomized group in the inflammatory phase on day 3 ($40.7 \pm 17.6\%$ and $21.0 \pm 16.8\%$, respectively). Therefore, the administration of daidzein under lack of estrogen is expected to reduce the inflammation period and promote re-epithelialization.

Key words: Daidzein, Ovariectomy, Female mice, Cutaneous wound healing, Inflammatory phase

Introduction

Recently, the reduction in estrogen that occurs at the menopause has been shown to have pronounced effects on skin and the cutaneous wound healing response.¹⁾ In postmenopausal women, delayed cutaneous wound healing is associated with increased inflammation, dysregulated protease activity, and reduced matrix deposition. On the other hand, exogenous estrogen treatment, either systemically or topically, reverses this delayed healing by reducing inflammation and stimulating matrix deposition and re-epithelialization.²⁻⁵⁾

Estrogen is a kind of ovarian hormone, and is active in the appearance of a woman's secondary sexual characteristics and adjustment of the periodical activity of sexual organs, for example, uterus and ovary. As hormone replacement therapy (HRT), estrogen has been prescribed for the symptomatic relief of subjective symptoms (e.g. hot flushes, night sweats, sleep patterns, mood changes, and vaginal dryness) in climacteric women, with an immediate improvement in quality of life. However, recently, The Women's Health Initiative⁶⁾ reported that HRT has side effects, such as increasing the risks of breast cancer and cardiovascular disease, so it searched for an alternative medicine as a substitute for HRT.

As one of these alternative medicines, isoflavone has attracted attention.⁷⁾ Isoflavone is a type of flavonoid that is abundant in soybean and has a molecular configuration similar to estrogen. In addition, it is a selective estrogen receptor modulator (SERM) with reported binding selectivity for β -estrogen receptor (ER) isoform⁸⁾, but with a binding affinity an order of magnitude lower than that of 17β -estradiol.⁹⁾ Genistein and daidzein belong to the group of isoflavones. Genistein injected intraperitoneally promotes wound healing by reducing inflammation and increasing matrix deposition.¹⁰⁾ Daidzein is metabolized by intestinal bacteria to produce equol and O-DMA, metabolites that are more estrogenic than daidzein.¹¹⁾ Moreover, genistein and daizein increase the moisture content of skin by stimulating hyaluronic acid.¹²⁾

We considered that oral administration of daidzein accelerates cutaneous wound healing when there is a lack of estrogen; therefore, the aim of the present study was to clarify the

effect of daidzein oral administration on cutaneous wound healing in ovariectomized mouse, a model of human age-associated delayed wound healing.

Materials and Methods

Animals

Twenty-three C57BL/6 female mice aged 8 weeks and weighing 17.7-19.0 g were used (Sankyou Lab Service Co., Tokyo, Japan). They were caged individually in an air-conditioned room at 25.0 ± 2.0 °C with light from 08:45 to 20:45 hours. Water was given freely and chow was given according to a protocol. The experimental protocol and animal care were in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University, Japan (AP-091218).

Wounding

Ten-week-old female mice that had undergone ovariectomy (OVX) 2 weeks previously were used for the experiments on wound healing. They were anesthetized with an intraperitoneal (IP) injection of pentobarbital sodium (0.05 mg/g weight), and the dorsum was shaved. Two circular (4 mm in diameter) full-thickness skin wounds including the panniculus carnosus muscle on both sides of the dorsum of the mouse were made with a Kai sterile disposable biopsy punch (Kai Industries, Gifu, Japan). Wounds were covered with hydrocolloid dressing (Tegasorb; 3M Health Care, Tokyo, Japan) to maintain a moist environment, and then the mouse was wrapped one and a half times with sticky bandages (Mesh pore tape; Nichiban, Tokyo, Japan). They were changed every day.

Feed and determination of the quantity of daidzein

The mice were divided into Groups A and B. All refined feed was custom-made by Oriental Yeast Co., Ltd. (Tokyo, Japan). Mice in both groups were provided with refined feed without daidzein for two weeks after ovariectomy. After wounding, Group A was provided with feed containing daidzein (Nagara Science Co., Ltd., Gifu, Japan) ad libitum, while Group B was provided with refined feed without daidzein (Fig. 1). Since we wanted to reflect healing promotion for a wound suffered by accident by a postmenopausal woman, we orally administered the daidzein right after wounding. Under our protocol, the quantity of feed that one mouse of 25 g in weight takes in one day is about 3 g. From the safe intake of

isoflavone of 70~75 mg/day/one man weighing 60 kg¹³⁾, isoflavone at 0.0012 mg/g weight/day for a man was calculated. Then, we converted the data for a man to a mouse: 0.0012 mg/g weight/day x 25 g weight of mouse ÷ 3 g feed intake/day = 0.01 g/g of feed. Therefore, 1 g of refined feed was set to contain 0.01 mg of daidzein; 10 kg of refined feed containing 100 ng was made by Oriental Yeast Co., Ltd.

Macroscopic observation

The day when wounds were made was designated as day 0, and the process of wound healing was observed from days 0 to 14 after wounding. Wounded edges were traced on polypropylene sheets and photographs were taken every day. The traces on the sheets were captured with a scanner onto a personal computer by Adobe Photoshop Elements 7.0 (Adobe System Inc., Tokyo, Japan), and the areas of wounds were calculated using image analysis software Scion Image Beta 4.02 (Scion Corporation, Frederick, Maryland, USA). Additionally, the body weight and intake of food were measured every day.

Histological observation

The mice were euthanized by a massive pentobarbital sodium IP injection on days 3, 7, 11, and 14 after wounding (Fig. 1). Three mice were sacrificed at each time point. The wounds and the surrounding intact skin were harvested, stapled onto transparent plastic sheets to prevent over-contraction of samples, fixed in IHC Zinc Fixative (BD PharmingenTM, Tokyo, Japan) for 24 h, and left wound samples were sectioned in the head-tail direction while those on the right were sectioned in the left-right direction. After these operations, the samples were dehydrated in an alcohol series, cleaned in xylene, and embedded in paraffin to prepare 5 µm serial sections. Sections of 5 µm thickness were stained with hematoxylin-eosin (H-E) or subjected to Azan staining, and immunohistologically stained with anti- α -smooth muscle actin (α -SMA) antibody (Dako Japan, Kyoto, Japan) for detecting myofibroblasts¹⁴⁾ or anti-CD31 antibody (Abcam, Cambridge, UK) for detecting blood vessels.¹⁵⁾ We measured the ratio of re-epithelialization (%) = length of new epithelium/length of wound between wound edges, number of new blood capillaries/mm² granulation tissue, number of

myofibroblasts/mm² granulation tissue, and ratio of collagen fibers in granulation tissue = number of pixels of collagen fibers/number of pixels of granulation tissue area using Adobe Photoshop Element 7.0 (CA, USA).

Statistical analysis

The data are presented as the mean \pm SD. Fodder intake, daidzein intake and wound areas, rate of epidermalization, rate of collagen fiber development, and the number of biogenesis blood vessels were compared using t-test with SPSS for Windows Version 10.1 (SPSS Inc. USA). Differences were considered significant at $P < 0.05$.

Results

Transition of weight and feed intake

The weights at the initiation of the experiment were as follows (Fig. 2): Group A 17.5 ± 1.3 g, Group B 19.1 ± 0.7 g. After the operation OVX and wound production, the weights of both groups decreased but then increased gradually thereafter. The weights at the end of the experiment were as follows: Group A 18.5 ± 1.7 g, Group B 19.7 ± 1.8 g. There was no significant difference between the two groups on any day. Mean feed intakes per day from day 0 to day 14 after the operation OVX were as follows: Group A 3.2 ± 0.2 , Group B 3.2 ± 0.9 . There was no significant difference between the groups. Mean feed intakes per day from day 0 to day 14 after wounding were as follows: Group A 2.8 ± 0.4 , Group B 3.1 ± 0.4 . There was no significant difference between the groups, but feed intake in Group A tended to be lower than that in Group B. In Group A, mean feed intake before wounding was significantly greater than that after wounding. In Group B, mean feed intake before wounding was almost the same as that after wounding. Mean intake of daidzein per day in Group A was 0.03 mg from day 0 to day 14 after wounding.

Macroscopic observations

The ratios of wound area on day 0 to day 14 to the initial wound area on day 0 were calculated (Fig. 3). Wound areas in both groups increased slightly after wounding. In Group A, wound area peaked on day 1 at 1.22 ± 0.10 compared with that on day 0 after wounding, namely, initial wound area, and decreased gradually to 0.42 ± 0.19 on day 8 and 0.16 ± 0.07 on day 14. In Group B, the wound area peaked on day 3 after wounding at 1.50 ± 0.35 compared with the initial wound area, and decreased somewhat rapidly to 0.49 ± 0.12 on day 8 and 0.15 ± 0.04 on day 14. There were no clear significant differences between the two groups on days 0 to 14. However, the wound area in Group A tended to be smaller than that in Group B during the inflammatory phase on day 4 after wounding (1.02 ± 0.32 and 1.48 ± 0.39 , respectively) and day 5 after wounding (0.85 ± 0.34 and 1.30 ± 0.25 , respectively).

In both groups, new epithelium was observed at the wound edge on day 3 after wounding

and covered the whole wound surface of all wounds on day 10 or 11 after wounding. Thereafter, all wounds ended in scar on day 14 after wounding.

Effect of daidzein on the progress of new epithelium, proliferation of collagen fibers, new blood capillaries, and myofibroblasts

On day 3 after wounding, the ratio of re-epithelialization, $40.7 \pm 17.6\%$, in Group A seemed to be greater than that of $21.0 \pm 16.8\%$ in Group B; although there was no significant difference between the groups, the ratio of Group A tended to be higher than the ratio of Group B (Fig. 4-1). After day 7, the ratios of re-epithelialization were almost the same in the groups (Fig. 4-1, a and b). On days 11 and 14 after wounding, new epithelium completely covered the wound surface in both groups.

On day 3, collagen fibers were thin and had a low density, but thereafter became thick with a high density along with wound healing (Fig. 4c and d). In Group A, the number of collagen fibers reached a maximum value on day 11, and that in Group B reached it on day 14, but there was no significant difference between the groups on days 3, 7, 11, and 14 after wounding (Fig. 4-2).

On day 3 after wounding, new blood capillaries formed in some sections in both groups, which extended from the wound edges. On day 7 after wounding, many new blood vessels were observed throughout the granulation tissue of all sections in both groups (Fig. 4e and f). In Groups A and B, they reached a maximum value on day 7 after wounding, and then decreased (Fig. 4-3). There was no significant difference between the groups on days 7, 11, and 14 after wounding.

On day 3 after wounding, a few myofibroblasts were observed along the wound edge in both groups (Fig. 4g). On day 7 after wounding, they increased rapidly and were observed throughout the granulation tissue (Fig. 4-4). In Group B in particular, a lot of myofibroblasts appeared in the wound bed and wound edge, and they formed bridge-like structures across the wound (Fig. 4h). Although there was no significant difference between the groups on day 7, the number of myofibroblasts in Group B tended to be larger than that in

Group A. After 7 days, they decreased dramatically. There was a tendency that the number of myofibroblasts in Group B was larger than that in Group A on days 11 and 14.

Discussion

It is known that a decrease of estradiol in ovariectomized rats increases food intake and that an increase of estradiol in ovariectomized rats exerts an inhibitory effect on food intake or results in the same food intake as in controls^{16, 17)}. Daidzein showed the same food intake effect as estradiol in this study because the food intake in ovariectomized mice that were provide daidzein after wounding tended to be lower than that in ovariectomized mice before wounding in Group A, and the food intake in ovariectomized mice before wounding was almost the same as that in ovariectomized mice without daidzein after wounding in Group B.

The rate of re-epithelialization in Group A, subjected to OVX with daidzein, tended to be higher than that in Group B, subjected to OVX without daidzein, on day 3 ($40.7 \pm 17.6\%$, $21.0 \pm 16.8\%$, respectively) in the inflammatory phase, although there was no significant difference between the two groups. The keratinocytes that form the cut edge of the wound begin to migrate within 24 to 48 hr¹⁸⁾. These cells dissect their way proteolytically through the fibrin-rich extracellular matrix, which covers the wound surface with plasmin and MMP (matrix metalloprotease)¹⁹⁾. Moreover, it has been shown that EGF (epidermal growth factor) binds to the ErbB family of receptor tyrosine kinases, resulting in cell proliferation, migration, and differentiation²⁰⁾. On the other hand, there have been no reports that daidzein and estrogen participate in the activity of plasmin or MMP; daidzein does not inhibit a tyrosine kinase like genistein, a tyrosine kinase inhibitor²¹⁾, and binds the estrogen receptor and causes cell proliferation like estrogen²²⁾. Therefore, the new epithelium in the daidzein-administered group may proliferate faster than that in the daidzein non-administered group in this study.

Moreover, we showed that the area in Group A, subjected to OVX with daidzein, tended to be smaller than that in Group B, subjected to OVX without daidzein, during the inflammatory phase 4 and 5 days after wounding, although there was no significant difference between wound areas of the two groups. Estrogen²⁻⁵⁾ and genistein, one of the isoflavones¹⁰⁾, have effects of decreasing the number of monocytes and macrophages and reducing wound area. Although we have not counted the number of inflammatory cells, our

results indicate that daidzein tends to reduce wound area in the inflammation phase, so probably wound area in the inflammatory phase is reduced by a similar pathway to that described above.

Myofibroblasts participate in wound contraction. Myofibroblast shrinkage with α -SMA transmits a contractile force through the fibronexus and intercellular adhesion molecules to the entire wound, thereby pulling the surrounding tissue centripetally to contract the wound.^{14,}

²³⁾ In our study, a few myofibroblasts in Groups A and B appeared on day 3 after wounding and they increased rapidly to spread throughout the granulation tissue by day 7. In Group B in particular, a lot of myofibroblasts formed bridge-like structures across the wound, as described by Tanaka et al.²³⁾ The number of myofibroblasts in Group B tended to be larger than that in Group A. Since the wound area compared with the initial area decreased from 1.22 on day 3 to 0.42 on day 8 in Group A with daidzein and from 1.50 on day 3 to 0.49 on day 8 in Group B without daidzein, wound contraction in Group B was greater than that in Group A. From this, we think that daidzein does not have an effect on myofibroblasts.

Age-related changes in skin are most prominent in postmenopausal women owing to their low estrogen levels. Studies have shown that elderly subjects heal more slowly than the young, and their wound healing is characterized by increasing inflammation, delayed re-epithelialization, delayed neovascularization, and reduced matrix deposition.^{24,25)} On the other hand, estrogen accelerates the cutaneous wound healing process, associated with enhanced matrix deposition, rapid epithelialization, and a dampening of the inflammatory response²⁻⁵⁾. Emmerson et al.¹⁰⁾ stated that genistein, one of the isoflavones, accelerates wound healing, affecting multiple aspects of the repair process, such as re-epithelialization and inflammation. As we have discussed, our research suggests that daidzein would promote wound healing of elderly subjects, affecting the inflammation phase, similar to estrogen and genistein.

Summary

Daidzein reduces the enlargement of wound area during the inflammatory phase and promotes re-epithelialization, the same as estrogen and genistein i.p. injection¹⁰⁾, although it does not shorten the wound healing period. This indicates that daidzein administration may be efficient for the control of inflammation.

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Legends

Figure 1. Experimental protocol. (\longrightarrow) Refined feed without isoflavone and (\dashrightarrow) refined feed containing daidzein (wall letters). Tissue harvest. (*) The day when wounds were made is designated as day 0.

Figure 2. Transition of weight. There were no significance differences between the two groups on any day. (◆) Group A, (■) Group B. n=4 in each group.

Figure 3. Graph showing the ratios (mean \pm SD) of wound area by day after wounding. There were no significance differences between Groups A and B on days 0 to 14. However, the wound area in Group A tended to be smaller than that in Group B during the inflammatory phase 4 and 5 days after wounding. (◆) Group A, (■) Group B. n=8 in each group.

Figure 4. (1): Ratios of new epidermis; black box is for Group A and white box Group B, n=5 in each group. (2): Ratios of collagen fibers in granulation cells; black box is for Group A and white box Group B, n=5 in each group. (3): New blood vessels; black box is for Group A and white box Group B, n=3-6 in each group. (4): Numbers of myofibroblasts; black box is for Group A and white box Group B, n=3-5 in each group. There were no significant differences between Groups A and B shown in all graphs.

(a, b): New epidermis almost covered the wound surface on day 7. Arrows in enlarged figures of quadrangles indicate the chips of elongating new epidermis. a is for Group A and b Group B. H-E staining. (c, d): The areas stained deep blue with Azan staining were filled with a lot of collagen fibers on day 11 after wounding. The collagen fiber density of Group A in c seemed to be higher than that of Group B in d. (e, f): There were many new blood vessels (arrows) stained by anti-CD31 antibody in granulation tissue of both Group A in e and Group B in f on day 11 after wounding. (g): There were a few myofibroblasts (arrows) stained with anti- α -SMA antibody along the wound edge of Group B on day 3. (h): A lot of myofibroblasts were observed in the wound bed and along the wound edge of Group B on day 7 after wounding. Note that they formed bridge-like structures (arrows) across the wound.

D: dermis, E: epidermis, G: granulation tissue, P: panniculus carnosus muscle. Solid line indicates the boundary between normal skin and wound, dashed line between dermis and

epidermis.

卵巣摘出雌マウスにおけるダイゼインの経口投与が皮膚創傷治癒におよぼす影響

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要旨

この研究は、卵巣摘出した雌マウスに皮膚創傷を作製し、経口投与したイソフラボン
の一種であるダイゼインが、創傷治癒にどのような影響を与えるかを観察したもので
ある。8週令の雌マウスを卵巣摘出群と卵巣摘出し創作製した後にダイゼインを与え
た2群に分けた。両群共、卵巣摘出後、ダイゼインを含まない精製飼料で2週間飼育
後に、左右の背部に直径4mmの皮膚全層欠損層を作製した。創作製後、ダイゼインを
含まない飼料とダイゼインを含む飼料で2週間飼育した。ダイゼインは、飼料1gに
0.01mg含むように作製した。両群の創面積は、2週間間の毎日において、有意差は
見られなかった。しかし、炎症期である創作製後4と5日では、ダイゼイン食で飼育
した群が無ダイゼイン食で飼育した群が、やや創面積が小さい傾向がみられた(それ
ぞれ、p値が0.061、0.083であった)。創作製後の3日での、再上皮化の割合は、

ダイゼイン群で $40.7 \pm 17.6\%$ 、無ダイゼイン群で $21.0 \pm 16.8\%$ となり、ダイゼイン群はより上皮化が進んでいる傾向が見られた ($p = 0.07$)。これらの結果は、エストロゲン欠乏状態で、ダイゼインの経口投与が、創傷治癒において、炎症を抑制し上皮化を促進することを示唆している。

キーワード：ダイゼイン、卵巣摘出、雌マウス、皮膚創傷治癒、炎症期

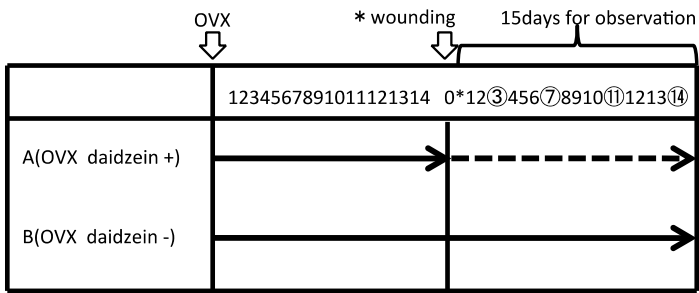


Figure 1

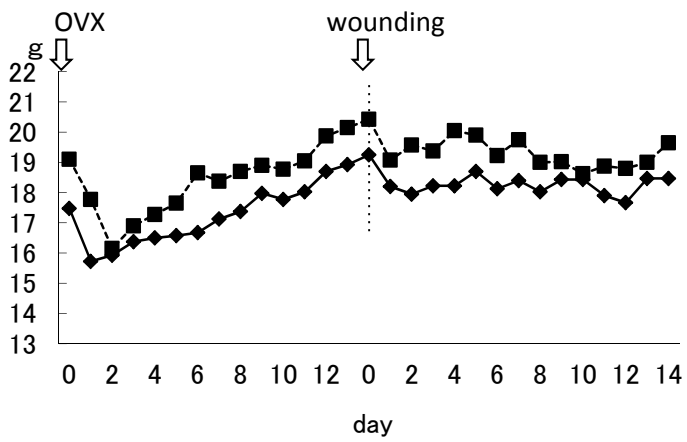


Figure 2

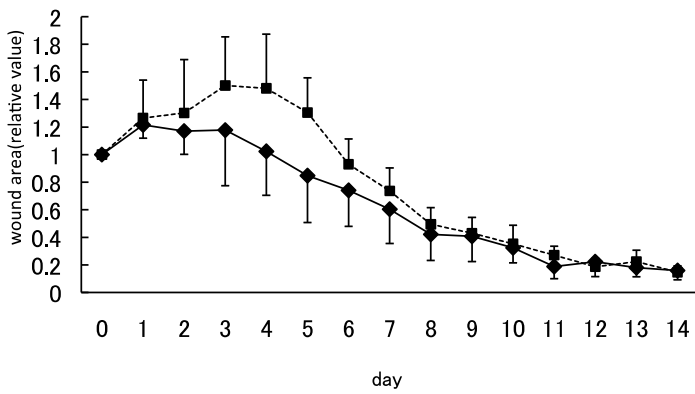


Figure 3

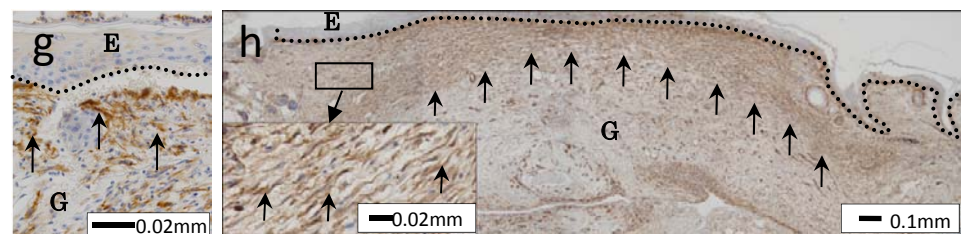
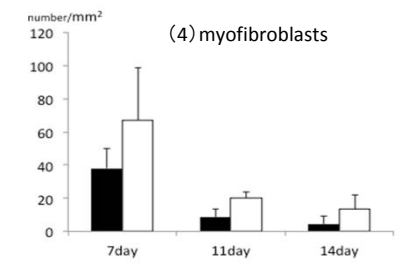
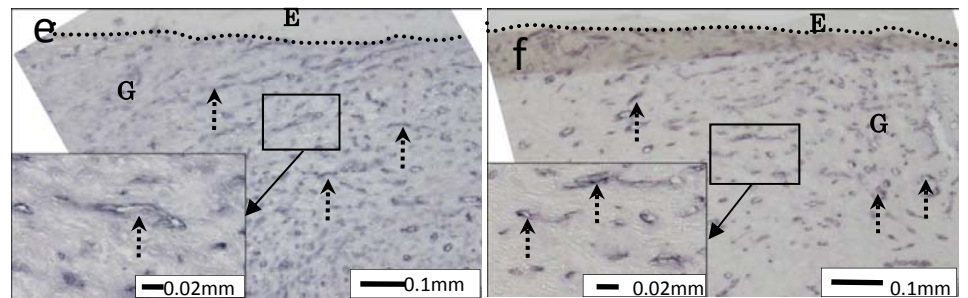
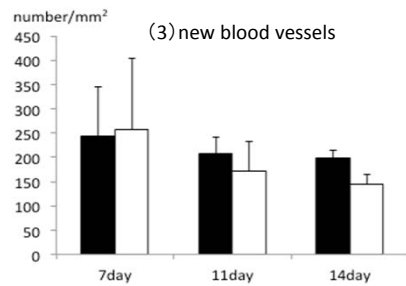
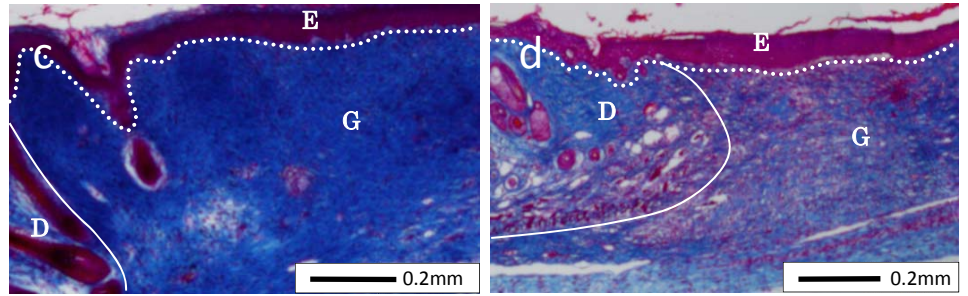
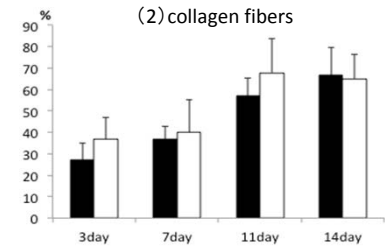
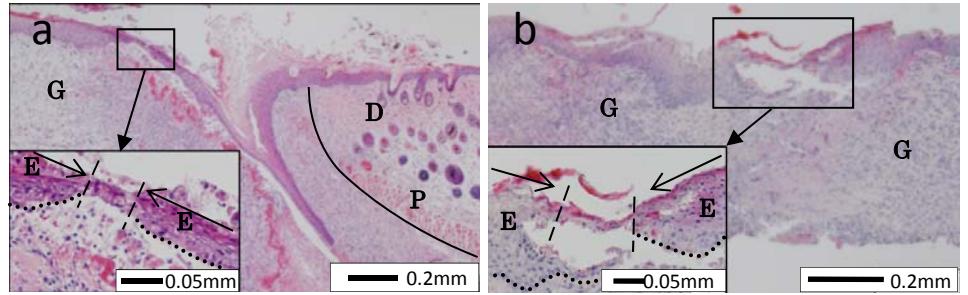
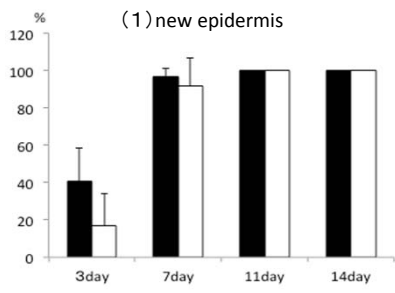


Figure 4