Enhanced wound healing by epigallocatechin gallate-incorporated collagen sponge in diabetic mice

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Abstract

Epigallocatechin-3-*O*-gallate (EGCG), the major polyphenolic compound present in green tea, has potent antioxidant and free radical scavenging activities. In this study, various concentrations (10, 100 and 1000 ppm) of EGCG were incorporated into a collagen sponge (CS) in order to investigate its healing effects on full thickness wounds created in type 2 diabetic mice. After 14 d, the residual wound size of the mice treated with 10 ppm EGCG-incorporated collagen sponge (E-CS) decreased significantly faster than that of the other mice. Moreover, significant increases in the degree of re-epithelialization, the thickness of the granulation tissue and the density of the capillaries were also histologically observed in the wound sites exposed to 10 ppm E-CS in comparison to the others. Furthermore, 10 ppm E-CS resulted in significant increases in the immunoreactivity of Ki-67 (re-epithelialization at wound site), CD31 (formation of blood vessels) and α-smooth muscle actin (the induction of myofibroblasts across the dermis). These results suggest that a CS incorporated with EGCG at low concentrations can enhance wound healing in diabetic mice by accelerating re-epithelialization and angiogenesis as well as improving the cellular reorganization of granulation tissue by triggering the activity of myofibroblasts.

INTRODUCTION

Wound healing is a complex multifactorial process involving the interaction of inflammation, re-epithelialization, angiogenesis, granulation tissue formation and collagen deposition. The wound healing process is associated with extensive oxidative stress to the system, which generally inhibits tissue remodeling. Epigallocatechin-3-O-gallate (EGCG), the predominant catechin from tea, possesses much stronger anti-oxidant activities than vitamin C by virtue of its peculiar stereo chemical structure and it has also been shown to play an important role in preventing cancer and cardiovascular diseases (1). Moreover, it also has other beneficial effects, including anti-inflammatory, antimicrobial and immunomodulatory activities (2). EGCG was also found to exert differential activities on normal epidermal keratinocytes vs. epidermoid carcinoma (A431) cells (3) and to induce the differentiation and proliferation of normal epidermal keratinocytes. These results suggest that EGCG can be used for the treatment of dermal wounds by means of facilitating re-epithelialization during the wound healing process (4).

Chronic wound healing is a troublesome and common complication of diabetes resulting in significant clinical morbidity. Genetically, diabetic mice are useful as an animal model for wound-healing studies, since wound healing in these animals is markedly delayed in comparison to nondiabetic littermates (5). Healing impairment is characterized by delayed cellular infiltration and granulation tissue formation, reduced angiogenesis and decreased collagen organization. The mechanism of this alteration is thought to result from the diabetic production of reactive free radicals that cause lipid peroxidation, which in turn impairs the activity of keratinocytes, endothelial cells and fibroblasts.

Collagen sponges (CSs) were transplanted to full thickness wounds to mimic the treatment of deep wounds in patients as closely as possible. The CS matrix promotes the

migration of host cells and blood vessels into the structure, thus allowing rapid replacement by host tissue at the wound site and finally helping to form dermis-like granulation tissue. However, the application of a CS only is limited to chronic wounds because the CS can easily degrade due to the lowered immunity of diabetic mice against microbial infection (5). This bacterial contamination triggers not only the degradation of the CS by collagenase produced from the bacteria, but also bacterial growth in the degraded CS (6). Therefore, it is necessary to improve the CS to overcome these problems. In this study, the wound healing effects of EGCG-incorporated collagen sponges (E-CSs) were investigated on full thickness wounds in type 2 diabetic mice. To evaluate the effects of E-CS, re-epithelialization, granulation, angiogenesis and myofibroblast induction were histologically or immunohistochemically examined at the wound sites. Since EGCG has a beneficial effect under high glucose diabetic conditions (7) and it also stabilizes collagen (8), EGCG may therefore be potentially used as a healing and supportive agent in diabetic wound healing utilizing a collagen-based artificial dermis.

MATERIALS AND METHODS

Animals

Genetically diabetic male BKS.Cg-+ $Lepr^{db}$/ +$Lepr^{db}$/JCL mice were obtained from Slc Japan Inc. (Osaka, Japan). All mice were maintained on a standard laboratory diet and water *ad libitum* and experimentally used when aged 10 wk old at the time of wounding. Each mouse was housed individually. Animal care followed the criteria of the Animal Care Committee of Institute for Frontier Medical Sciences, Kyoto University for the care and use of laboratory animals in research.

E-CS preparation and surgical procedures for wounding

E-CSs were prepared by treating CSs (Pelnac®, Gunze Co., Kyoto, Japan) with various concentrations (10, 100 and 1000 ppm) of EGCG (Teavigo™, DSM Nutritional Products Ltd., Basel, Switzerland) overnight at 4°C. The diabetic mice were anesthetized with intraperitoneal pentobarbital (30 mg/kg, Nembutal, Abbott Laboratories, North Chicago, IL). Under sterile conditions, the dorsal area was totally depilated and a single full thickness excisional square wound (1×1 cm^2) was created on the upper back of each mouse using a pair of sharp scissors and a scalpel. Either CSs or various concentrations of E-CSs were transplanted into the wounds of the mice. In order to observe the direct effect of EGCG on wound healing, the mice topically received 0.1 ml of EGCG (10, 100 and 1000 ppm, respectively) or vehicle (saline) solution once a day for 3 d after wounding. After 7 and 14 d of the transplantation of CS or E-CSs, the mice were euthanized using pentobarbital anesthesia. All experiments related to surgical procedures and treatments were performed in accordance with the guidelines of the Animal Experiment Committee of Institute for Frontier Medical Sciences, Kyoto University.

Determination of residual wound area

In order to determine EGCG-mediated healing efficiency, the residual wound size was measured from the unclosed wound area after 7 and 14 d of transplantation using a digital planimeter (PLACOM KP-90N, Koizumi Sokki Mfg. Co. Ltd., Nagoya, Japan). After wound size measurement at 7 and 14 d, the wounded tissues were excised in full depth and bisected. The tissues were fixed in 10% formaldehyde and then embedded in paraffin for the

histological and immunohistochemical evaluations as mentioned below.

Histological and immunohistochemical analyses

Paraffin-embedded tissue blocks were sectioned by a 5 μm thickness and then stained with hematoxylin and eosin (H&E). The sections were also stained with rabbit polyclonal antibody Ki-67 (Novocastra, Newcastle, UK), mouse monoclonal antibody α-smooth muscle actin (α-SMA, clone 1A4, Dakocytomation, Inc., Carpinteria, CA) and mouse monoclonal antibody CD31 (PECAM-1, Novocastra). To immunohistochemically examine the expression of Ki-67 and CD31 in the excisional wound tissue, the sections were pretreated in 10 mM citric acid buffer (pH 6.4) at 121°C for 20 min and then washed with PBS. To block endogenous peroxidase, all sections were immersed in 10% H_2O_2 in methanol for 10 min and then washed with PBS for 5 min. To determine myofibroblasts, α-SMA was detected by incubating dewaxed sections first in 10% rabbit serum for 30 min, followed by mouse anti-human α-SMA antibody at 4°C overnight. The bound antibody was detected by biotinylated rabbit anti-mouse antibody and streptavidin–HRP–DAB. Negative controls were generated by incubating slides with immunoglobulin from the same species and at the same final concentration, but with no primary antibody. The prepared sections were all observed under an optical/fluorescence microscope (Biozero – 8000, Keyence, Osaka, Japan).

Histomorphometric and quantitative analyses

The H&E-stained sections as prepared above were histomorphometrically examined for the epithelial regeneration and organization of granulation tissues. For measuring the length

of re-epithelialization, the wound edges were determined as the border between the original dermis with hair follicles and the newly developed fibrotic dermis without hair follicles. The thickness of granulation tissue was determined, at the center of each section, from the surface of granulation tissue to the margin of dermis and subcutis, vertically.

To determine the number of capillary and immuno-reactive cells, the stained wound sections were observed under $\times$ 200 magnifications with a test grid eyepiece. The number of Ki-67 positive cells at the basement membrane of epidermis was counted in both wound margin areas (300 $\times$ 300 μm^2 in dimension). The number of α-SMA and CD31 positive cells was measured in three different areas ($\times$ 200 magnifications) at both ends as well as in the middle of dermis. These quantitative counts were made independently by two observers blinded to treatment, and the counts were compared to ascertain that no more than 10% difference existed between counts from the two observers on the same tissue sections.

Statistical analysis

All variables were tested in four independent experiments, and each experiment was repeated three times (n = 10-12). Quantitative data were expressed as mean $\pm$ standard error. Statistical comparisons were carried out with an analysis of variance (ANOVA, SAS Institute Inc., Cary, NC), which was followed by the Fisher's PLSD test using StatView-J 4.5 software package (Abacus Concepts, Inc., Berkeley, CA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Effect of E-CS on the cutaneous wound healing in diabetic mice

Figure 1 shows the appearance of the wounds after 7 and 14 d of treatment. After 7 d, the connective tissues were observed to have slightly regenerated with some local infection. However, at 14 d 10 ppm E-CS treatment resulted in an appreciable improvement in the wound closure in comparison to that observed with the CS only (control) and other concentrations of E-CSs. In addition, the wounds treated with 10 ppm E-CS decreased in size without oozing. The residual wound area was also determined by measuring the unclosed wound area as a function of time (**Fig. 2**). The wounds began to close after 10 d and the residual wound sizes significantly decreased in all groups after 14 d. Importantly, the wounds treated with 10 ppm E-CS showed the smallest residual wound area. In contrast, the mice treated with 1000 ppm E-CS showed impaired wound closure. The residual wound area in the CS treated group was intermediate which is similar to the wound size of the 100 ppm E-CS treated group.

Effect of E-CS on re-epithelialization and granulation formation

To examine the effects of EGCG on epithelial and dermal regeneration, the length of re-epithelialization and the thickness of the granulation tissue were determined, respectively. **Figure 3A** shows the length of the regenerated epidermis in the wounds treated with CS or various concentrations of E-CSs. There was no significant difference in the epithelial regeneration between the wounds treated with CS and E-CSs at 7 d (**Fig. 3B**). However, the re-epithelialization significantly ($p < 0.05$) increased in the wounds treated with 10 ppm E-CS after 14 d. These results were consistent with those of the residual wound area. Moreover, 10 ppm of E-CS treatment resulted in a significant ($p < 0.05$) enhancement in the granulation tissue formation (**Fig. 4**). However, after 7 d of treatment, no significant

difference was observed in the granulation tissue formation between the wounds transplanted with CS and E-CSs (Data not shown). The wounds treated with 10 ppm E-CS showed a well-regenerated and differentiated epidermis, relatively higher cell number and significantly thick dermis, whereas those treated with CS showed early on-going epithelialization, evident edema and poorly formed granulation tissue (**Fig. 3A** and **4A**). Furthermore, it was also revealed that 10 ppm E-CS induced an increase in the epithelial migration with a concomitant decrease in the wound gap and width after 14 d of transplantation. These results indicate that the 10 ppm E-CS treatment significantly accelerates the epithelial and dermal regeneration of the diabetic wound site

Immunohistochemical analysis of the effect of E-CS on wound healing

Figure 5 shows the representative immunolocalization of Ki-67, α-SMA and CD31 in the wounds treated with CS or E-CSs after 14 d. Ki-67-positive cells were mainly observed at the basal layer of the epidermis (**Fig. 5A**). The strongest positive staining for Ki-67 was shown in the wounds treated with 10 ppm E-CS after 14 d; thus leading to significantly ($p <$ 0.05) more newly formed epidermis with a thicker layer of the basal membrane. This result implies that re-epithelialization in the wounds treated with 10 ppm E-CS was enhanced by increased proliferation of basal epidermal keratinocytes. It was also found that the number of cells positive for Ki-67 in the 10 ppm E-CS treated group was significantly ($p < 0.05$) higher than that in the others (**Fig. 5B**).

α-SMA-positive myofibroblasts across the dermis were mainly observed at the bottom of granulation tissue as shown in **Figure 5A**. It was revealed that treatment with 10 ppm E-CS resulted in significant ($p < 0.05$) increase in the number of cells expressing α-SMA after 14 d (**Fig. 5B**). However, the other wounds treated with 100 or 1000 ppm E-CS showed

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fewer α-SMA-positive stained cells than for the CS transplantation.

The wounds treated with 10 ppm E-CS showed the greatest positive staining for CD31 and significantly ($p < 0.05$) more newly generated capillaries, which were diffusely located and clearly formed tube-like structures (**Fig. 5A**). The size of vessels in the 10 ppm E-CS treated wounds was much bigger than that in the others. On the other hand, the CD31-positive stained capillaries in 100 or 1000 ppm E-CS treated wounds were almost identical to those in the CS treated group. There was no significant difference among them; however, the capillary formation was slightly enhanced in the wounds treated with 1000 ppm E-CS (**Fig. 5B**).

DISCUSSION

Kapoor *et al*. demonstrated that epicatechin gallate (ECG), one of the catechin components in green tea, could significantly improve the quality of wound healing and scar formation in an incisional wound healing model in rats (9). These reports led us to investigate the effect of CSs incorporated with EGCG on chronic wound healing. Although EGCG is considered to be a potentially effective wound-healing agent with a wide range of pharmacological and biological activities, relatively little is known about the precise mechanisms of the wound-healing effect of EGCG *in vivo*.

Clinically, it is important to reduce the wound area as fast as possible in order to alleviate stress as well as reduce the possibility of infection and dehydration (10). In addition, the measurement of the residual wound size is a simple and frequently utilized way to analyze the efficiency of any treatment on wound healing. As shown in **Figures 1** and **2**, 10 ppm E-CS treatment accelerated wound size reduction in comparison to the control and other groups. This was associated with both accelerated re-epithelialization and contraction (**Fig. 3**).

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Epithelialization is achieved by the proliferation and migration of epidermal cells from the wound edge. Wound contraction is based on a full-thickness afferent shift (11) and it favorably accelerates wound healing, but it can also sometimes cause a functional disturbance. Therefore, an appropriate balance of epithelialization and wound contraction is required for this region.

EGCG has been shown to stimulate the proliferation and differentiation of keratinocytes (4). Keratinocytes are the major cell type of the epidermis and they play an important role in epidermal construction (12). The basal epidermal layer contains keratinocyte stem cells that replenish the epidermis (12). Ki-67 is a nuclear protein that is expressed in proliferating cells and widely used as a proliferation marker (13). In the present study, 10 ppm E-CS treatment significantly accelerated re-epithelialization via the proliferation of basal epidermal keratinocytes, which was demonstrated by Ki-67 immunostaining (**Fig. 5**A).

Wound size reduction in the wounds treated with 10 ppm E-CS was also confirmed by an appreciable enhancement in the number of α-SMA expressing myofibroblasts in the dermis (**Fig. 5**). During granulation tissue formation, fibroblasts differentiate into myofibroblasts (14). The new connective tissue is synthesized by myofibroblasts, fibroblasts capable of migrating and characterized by the presence of contractile features due to their intermediate morphology between that of a fibroblast and a smooth muscle cell. The presence of myofibroblasts is considered to be characteristic of tissue undergoing contraction (15, 16). The apparent greater number of myofibroblasts in the 10 ppm E-CS treated group may be partially responsible for the fast wound contraction. Therefore, it is suggested that the reduction of wound size in 10 ppm E-CS treated group might be attributed to the coordination of re-epithelialization and contraction mediated by increased activation of epidermal keratinocytes and myofibroblasts, respectively. Therefore, these results show that a collagen sponge incorporating 10 ppm of EGCG is therefore promising for future clinical application.

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The fact that wound reduction observed in the E-CS treated group at 7 d was poor in comparison to that on day 14 may be partially explained by EGCG having a potent mitogenic and chemoattractive effect of EGCG on endothelial cells and fibroblasts. In other words, EGCG is likely to mainly promote the second half of the wound healing process, including granulation, angiogenesis and re-epithelialization, which is therefore an important factor in wound reduction. In fact, in the present study, granulation, vascularization and epithelialization were all significantly accelerated by 10 ppm E-CS treatment on day 14. Figure 4 indicates thick granulation tissue with both fibrous tissues and cellular components to be closely related to re-epithelialization, angiogenesis and wound reduction. In addition, the enhanced capillary formation and re-epithelialization were confirmed by an immunohistochemical analysis (**Fig. 5**).

In contrast, treatment with 1000 ppm E-CS did not enhance re-epithelialization (**Fig. 1-3**). The topical and repeated exposure to EGCG could thus lead to dermal sensitivity and minor irritation (17). The impaired healing observed in the 1000 ppm E-CS treated group could be the result of the toxicity of EGCG when administered at high doses.

The data are not shown but the LD50 value of EGCG was investigated in various cell types. Human microvascular endothelial cells were highly sensitive to EGCG. Although the species was different from that of the current study, the LD50 value of 70 ppm in endothelial cells implies that 100 ppm EGCG treatment might be cytotoxic to endothelial cells and thus inhibit angiogenesis.

Newly formed granulation tissues were found to be substantially thick and well developed in the wounds treated with 10 ppm E-CS (**Fig. 4**). Granulation tissue formation was not expected to be induced by application of EGCG because EGCG is reported to inhibit fibroblasts migration *in vitro* (18). However, EGCG treatment with a low dose, i.e.10 ppm, was shown to enhance the organization of granulation tissue by stimulating the growth of

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fibroblasts in the present study. Stimulation of skin fibroblasts can contribute to the fibroblast-keratinocyte-endothelium complex that not only repairs wounds, but also maintains their integrity. In the highly coordinated biological process of dermal wound healing, skin fibroblasts interact with surrounding cells and produce collagen-based extracellular matrices which ultimately replace the provisional fibrin-based matrix and accelerate the reduction of the wound edges through their contractile properties (15).

Wound healing is an angiogenesis-dependent process, because oxygen and nutrients are required to promote the newly forming granulation tissue. An immunohistochemical analysis using the endothelial cell marker, CD31 showed that new capillaries were significantly regenerated in wounds treated with 10 ppm E-CS (**Fig. 5**). During the regeneration process, revascularization can increase the resistance to infection (5). In diabetic mice, an inadequate blood supply that results from venous insufficiency and cuffing of microvessels are contributing factors toward healing impairment. In this study, EGCG was shown to exert a potent angiogenic activity *in vivo*.

In conclusion, although the mechanism responsible for the acceleration of wound healing in diabetic mice is still not completely understood, the results of these experiments demonstrate that transplantation of a CS incorporated with a low concentration of EGCG induced significant wound contraction, re-epithelialization and angiogenesis as well as the reorganization of granulation tissue by triggering the activity of myofibroblasts.

EGCG has been shown to activate different pathways depending on the cell type while functioning differently according to its concentration (19, 20). T. Yamamoto *et al*. showed 200 μM (91.6 ppm) of EGCG damaged only tumor cells (oral squamous cell carcinoma, OSC-2 and OSC-4) but not normal cells (normal human primary epidermal keratinocytes). Chen *et al*. have reported that exposure to 200 μM (91.6 ppm) EGCG for 8 hr induced less than 1% apoptosis in WI38 human fibroblasts. *In vitro* studies have indicated the mechanism

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of EGCG activity at the cellular level. However, the wound healing process *in vivo* is complicated and multifactorial and it is hard to elucidate the mechanism of EGCG involvement. Although the overall beneficial effects of EGCG *in vivo* were shown in this study, further investigation of the wound healing mechanism of EGCG is necessary before clinical use.

Abbreviations

Collagen sponge   CS

EGCG-incorporated collagen sponge   E-CS

Epigallocatechin-3-O-gallate   EGCG

Hematoxylin and eosin   H&E

α-smooth muscle actin   α-SMA

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FIGURE LEGENDS

Figure 1. Appearance of the wounds in diabetic mice after 7 and 14 d of treatment of CSs incorporate without or with various concentrations of EGCG. These photographs are representative of 10 independent experiments, showing similar results.

Figure 2. Residual wound size of diabetic mice treated with CS (EGCG 0 ppm) or various concentrations of E-CSs. It was measured from the unclosed wound area after 7 and 14 d of treatment using a digital planimeter. The results are reported as mean ± standard error (n = 10) and analyzed by the Fisher's PLSD test. The value marked with an asterisk is significantly ($p < 0.05$) different from the control (CS).

Figure 3. Effect of E-CSs on the epithelial regeneration. A) Histological observations ($\times$ 50) of the regenerated epithelial tissues in the wounds treated with CS or various concentrations of E-CSs after 7 and 14 d. Dotted lines indicate the leading edge and route of re-epithelialization where an arrow ($\uparrow$) on the left side of each photograph represents the wound edge, and another arrow ($\downarrow$) on the right side represents the end point of re-epithelialization. These photographs are representative of 8 independent experiments, showing similar results. B) The length of re-epithelialization. The results are reported as mean ± standard error (n = 10) and analyzed by the Fisher's PLSD test. The values marked with an asterisk are significantly ($p < 0.05$) different from those after 7 d. The value marked with a sharp is significantly ($p < 0.05$) different from the other groups.

Figure 4. Effect of E-CSs on the formation of granulation tissues. A) Histological

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observations ($\times$ 100) of the newly formed granulation tissues in the wounds treated with CS or various concentrations of E-CSs after 14 d. Dotted arrows indicate the center of granulation tissues. These photographs are representative of 8 independent experiments, showing similar results. B) The thickness of granulation tissue. The results are reported as mean ± standard error (n = 10) and analyzed by the Fisher's PLSD test. The value marked with an asterisk is significantly ($p < 0.05$) different from the other groups. The value marked with a sharp is significantly ($p < 0.05$) different from the control (CS).

Figure 5. Effect of E-CSs on the wound healing in diabetic mice. A) Immunohistochemical observations ($\times$ 200) of the wounds treated with CS or various concentrations of E-CSs and stained with anti-Ki-67, anti-α-SMA and anti-CD31 antibody after 14 d (A scale bar means 100 μm). These photographs are representative of 8 independent experiments, showing similar results. B) Quantitative analyses of cells showing immunopositivity to Ki-67, α-SMA or CD31. The results are reported as mean ± standard error (n = 10) and analyzed by the Fisher's PLSD test. The value marked with an asterisk is significantly ($p < 0.05$) different from the other groups. The value marked with a sharp is significantly ($p < 0.05$) different from the control (CS).
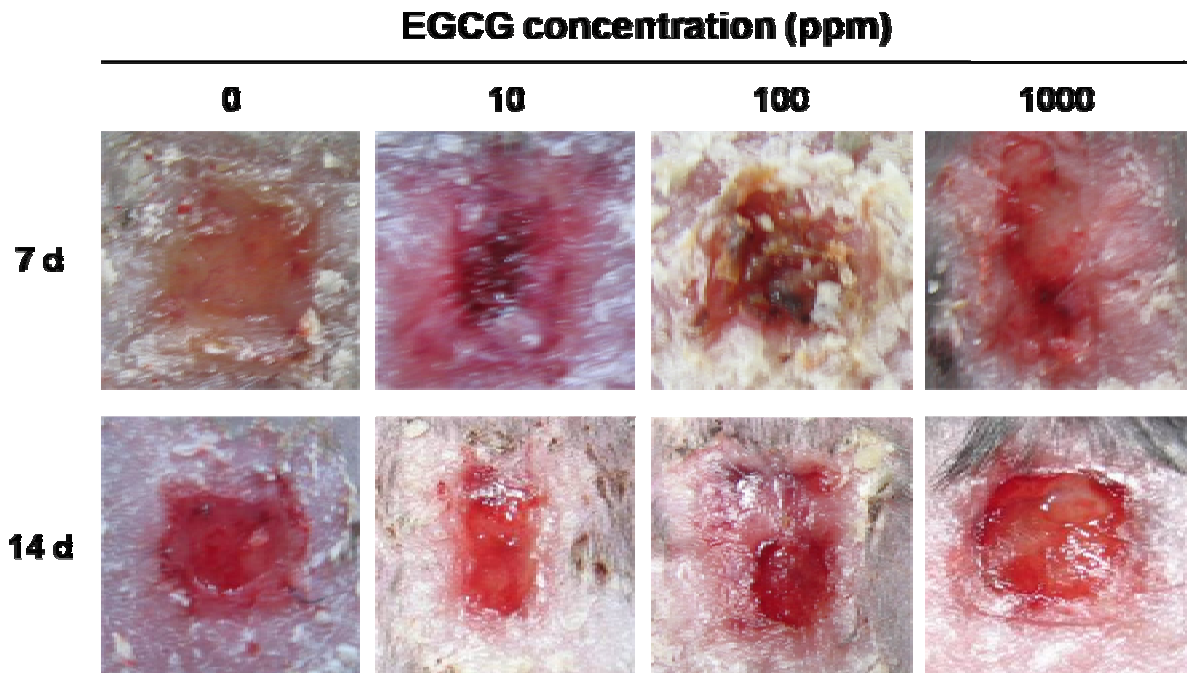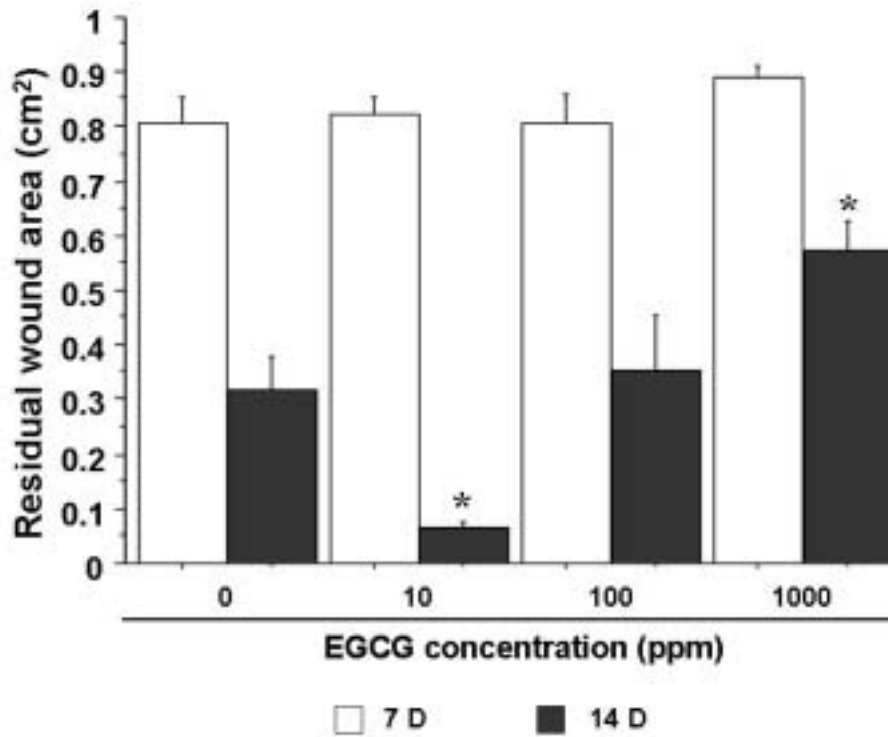
Fig. 1

EGCG concentration (ppm)

	0	10	100	1000

Fig. 2

Figure 2. by HH Kim et. al.

Fig. 3

(A)

(B)