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Liposome-encapsulated farnesol accelerated tissue repair in third-degree burns on a rat model

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ABSTRACT

Third-degree or full-thickness burns refer to lesions that extend to the epidermis, dermis, and subcutaneous tissue. The pathophysiology of burn wounds is characterized by tissue inflammation, edema, and hypertrophic scarring. Farnesol is a natural 15-carbon organic compound that possesses many biological effects. We have previously demonstrated that farnesol gel exerts restorative actions on ultraviolet B (UVB)-caused sunburn in vivo. The in vitro results revealed that liposomal farnesol from 0.04 mM to 0.8 mM significantly enhanced collagen production by murine skin fibroblasts, whereas liposomal farnesol at high (0.8 mM) and low concentration (0.04 mM) did not show any suppressions on skin fibroblast proliferation. We treated third-degree burns on a rat model with a formulated gel composed of various ratios of 2% hydroxypropyl methylcellulose (HPMC) and 4 mM liposomal farnesol for 7 and 14 days. On days 7 and 14 post wounding, histopathological observations revealed that the HPMC:farnesol gel ratios of 1:2 and 2:1 exerted the greatest tissue-repairing effects on the skin after third-degree burns compared with skin untreated or treated with a commercial burn gel and HPMC alone. These findings were consistent with the in vivo quantitative collagen-producing assay, wound healing scoring, and IL-6 Western blot results. These findings demonstrated that the fabricated liposomal farnesol gel is potentially able to promote wound healing after third-degree burns.

1. Introduction

Burns are a common injury in the modern society and a global health concern accounting for an estimated 180,000 deaths every year. Domestic and industrial accidents are the major causes of burns [1]. In the United States, 486,000 patients received medical treatment for burn injuries each year, with nearly 40,000 hospitalization related to burns. Two key therapeutic approaches are used to encourage restoration from burns: prevention of infection and stabilization of the patient. These are highly important concerns clinicians must consider when assessing the goals of outpatient burn management [2,3].

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In addition, for acceleration of wound healing and functional recovery, the first therapeutic approach is halting the instant inflammation triggered by burns, and the second approach is facilitating wound healing [4]. Traditionally, silver sulfadiazine (SSD) cream is the first-line therapy for minor burn wounds, but it has been shown to be outdated because of its side effects, such as neutropenia, erythema multiforme, crystalluria, and methemoglobinemia [5,6]. Herbal extracts, such as Robacin (which contains herbal components including: *Rosa damacena*,*Calendula officinlis*, and beeswax), aloe vera extracts, and moist exposed burn ointment (MEBO, which contains six herbal extracts and has an active ingredient b-sitosterol in a base of beeswax and sesame oil), in particular, have been demonstrated to possess angiogenesis- and fibrosis-decreasing, inflammation-inhibitory and wound healing-promoting effects [7]. Thus, compounds and extracts from natural products that have wound healing actions have provided alternatives for regeneration of healthy and functional skin [8].

Farnesol is an organic 15-carbon sesquiterpene alcohol produced by *Candida albicans* that possesses numerous biological actions. Farnesol is available from many natural sources including vegetables, fruits, and herbs (such as peaches, tomatoes, corn, lemon grass, and chamomile) [9,10] and it has been found to have antimicrobial, antiproliferative, antiallergic, anti-inflammatory, and tumor-related apoptosis-inducing effects [11–14]. Previous investigation revealed that farnesol increases keratinocyte differentiation via peroxisome proliferator-activated receptor (PPAR) [15]. Partially through this pathway, farnesol was demonstrated to improve metabolic abnormalities in vitro and in vivo [16]. Furthermore, farnesol may have positive actions on wound healing because it has been shown to reduce oxidative stress, inflammation, and apoptosis in 1,2-dimethylhydrazine-induced damage in the colon of Wistar rats. Moreover, cytokine regulation-associated anti-inflammatory or antiallergic effects on ovalbumin-sensitized and -challenged asthmatic mice in vivo have been revealed. Notably, Ku et al. demonstrated that farnesol downregulates the essential inflammatory cytokines such as interleukin (IL)-1beta, IL-6, tumor necrosis factor (TNF)-alpha, and TNF-alpha/IL-10 ratio in vivo. The normal functions of skin repair and wound healing attained and maintained by the balance of its paracrine system, which is contributed by the dermal fibroblasts and epidermal keratinocytes. Moreover, cell–cell and cell–matrix interactions are extremely essential in all phases of tissue repair. These interactions are manipulated and regulated by various cytokines and growth factors. Moreover, topical administration of certain anti-inflammatory cytokines, such asIL-8 and platelet-derived growth factor, despite inconsistent effectiveness, or agents, such as emu oil, that suppresses the production of proinflammatory cytokines, have been found to promote wound healing [17]. Thus, these findings suggest that farnesol is capable of modulating connective tissue and extracellular matrix synthesis and wound healing in the skin.

Lipid-based nanoparticle-constituted drug carriers have played a major role in the drug delivery system. One of the major benefits of drug carriers is to extend the effects of drugs. In addition, drug carriers made of liposomes are the least biologically toxic and relatively cheap. Furthermore, liposomes are primarily composed of phospholipids, which are the main components of biological membranes. These crucial characteristics make liposomes one of the most approved formulations in clinical use to treat cancer and infectious diseases [18]. We have previously shown that liposomal encapsulation in combination with iontophoresis in vivo enables beta-blocker propranolol to exert antiosteoporotic effects at a dose lower than its clinical therapeutic range [19]. We also found that farnesol gel possesses ultraviolet B (UVB)-screening capacity and exerts reparative effects against UVB caused sunburns [20]. However, severe damage to skin, such as third-degree burns, requires longer and more stable wound healing-promoting effects than those for sunburns. Drug carriers, in particular lipid-based nanoparticles such as liposomes, possess unique features of slow and sustained drug release, increased stability of encapsulated drug and improved pharmacokinetic effects, and therapeutic index of drugs. Therefore, the current study investigated for various formulations and ratios of liposome-encapsulated farnesol and HPMC applied to third-degree burns in a rat model. The liposomal farnesol and HPMC fabricated at two different ratios exerted obvious reparative effects on third-degree burns, demonstrating that liposomal farnesol with HPMC could be a feasible therapeutic formula for severe burns.

2. Materials and methods

2.1. *Preparation of liposomal farnesol*

Liposomes were fabricated using the evaporation-sonication method described previously with some modifications [21]. Briefly, DSPC and cholesterol were mixed at a molar ratio of 8.9:2.7. DSPC in powder form and cholesterol were dissolved in methanol: chloroform (1:3, v/v). Farnesol was then added at various concentrations to prepare liposomal farnesol. The morphology was observed through transmission electron microscopy (Tecnai G2, USA) and the size of liposomal farnesol was determined by Nanoparticle Tracking Analyzer (ZetaView NTA, Germany).

2.2. *Drug encapsulation efficiency and in vitro drug release*

The prepared farnesol-loaded liposomes were centrifuged at 12,000 rpm for 20 min while the free farnesol in the supernatant was qualified and quantified through high-performance liquid chromatography (Agilent 1100 series, USA). The EE% of the farnesol was calculated using the formula: EE (%) = (ADa/ADb) × 100%, where ADa is the amount of farnesol in the liposome found after centrifugation. ADb is the amount of farnesol in liposome found before centrifugation. The farnesol release rate from liposomes was measured by calculating the free farnesol in the supernatant at each predetermined time. In vitro release (%) = [(total amount of farnesol-residue of farnesol)/total amount of farnesol] × 100%.

2.3. *Cytotoxicity of liposomal farnesol*

Cell Counting Kit-8 (CCK-8; Sigma, USA) assay was used to assess the cytotoxicity of liposome-encapsulated farnesol on L929 fibroblasts according to the manufacturer's instructions. The fibroblasts were seeded on 96-well plates and then

exposed to liposomal farnesol at different concentrations for 24h. Ten microliters of CCK-8 reagent was added to each well, and the plate was incubated at 37°C for 2h. The absorbance of each well was read on a multiplate reader at 450nm.

2.4. Determination of collagen production in vitro

Collagen production was quantified using an assay reported in our previous study [20]. Briefly, L929 fibroblasts (1.5×10^5 cells/mL) were seeded in a 6-well plate for 24h. The fibroblasts were then exposed to treatments in the presence or absence of pure farnesol or liposomal farnesol for 24 or 48h. The amount of collagen was determined using the Sircol Soluble Collagen Assay Kit (Biocolor, Carrickfergus, Northern Ireland). The samples were incubated in an isolation and concentration reagent (polyethylene glycol in a TRIS-HCl buffer, pH 7.6) at 4°C overnight. The collagen in each group was precipitated and stained using the Sircol Dye Reagent, and the products were washed with Acid–Salt Wash Reagent. After centrifugation and resuspension in the alkali reagent, the absorbance was measured at 555nm on a multiplate reader.

2.5. Establishment of the rat model of third-degree burns

A total of 45 adult male SD rats weighing 150–200g were used for establishing a model of third-degree burns for therapeutic experiments. The in vivo procedures and the animal use protocol conducted in this study have been approved by the Institutional Animal Care and Use Committee of I-Shou University (approval number: AUP-105-50-02 and AUP-106-50-01). Nine adult male SD rats were used to evaluate and establish the third-degree burns on a rat model. Contact time for 4 (N=3), 6 (N=3) or 8 (N=3)s was respectively applied to the rat skin and the level of burns was assessed. Consistently, 8s contact of the 400°C heated soldering iron caused third-degree burns in the skin of 3 rats according to the depth and severity of burns. Thirty-six SD rats, classified into 6 groups (3 rats evaluated on day 7 and 3 assessed on day 14 post burn), were used in the evaluation of therapeutic effects of liposomal farnesol on the established third-degree burns in the rat burn model. The rats were reared in a temperature and humidity maintained room and raised on a 12-h light–dark cycle (lights on at 6:00 AM). Food and water were offered ad libitum during the experiment. After deep anesthesia using Zoletil® (40mg/kg, i.p.) and xylazine (10mg/kg, i.p.), third-degree burns ($2 \times 2 cm^2$) were created on the back skin of SD rats by placing the tip of a heated (400°C) soldering iron in contact with the skin of the thigh for 8s each. The rats were administered cephalexin (15mg/kg, SC, bid) and ketorolac (10mg/kg, IM, q24h) for 3 days to prevent the infection and suppress the pain. The activity, behavior, and appetite of the rats were carefully monitored twice per day, and the weight of the rats was measured twice a week by a veterinarian.

2.6. Histopathological analysis

2.6.1. Hematoxylin and eosin staining

Skin areas of third-degree burns administered with or without prepared gels or commercial cream were fixed in formalin and embedded in paraffin. The skin specimens were cut into 4-μm-thick sections. H&E staining was conducted in staining dishes. First, dewaxing was performed in xylene. Skin tissues were sequentially rehydrated with graded ethanol and tap water. Harris' H&E Y was then used to stain the skin tissues, followed by a wash in tap water for 30min. The skin tissues were then dehydrated with incubations ethanol and xylene.

2.6.2. Masson's Trichrome staining

Masson's Trichrome staining was executed for further analysis of burns in the skin. The skin specimens were deparaffinized in xylene and then hydrated in graded ethanol, xylene, and distilled water. Weigert's iron hematoxylin solution was applied to stain the skin tissues. Afterward, tissue slides were sequentially incubated in Biebrich scarlet-acid fuchsin (Sigma, USA) for 5min, followed by a 10-min treatment with phosphomolybdic–phosphotungstic acid solution and a 5-min treatment with aniline blue (Sigma).

2.6.3. Histopathological scoring of postburn wound healing

The wound healing efficacy after third-degree burns was further assessed using histopathological scoring based on the previous methods with some modifications [22,23]. Briefly, the scores of epithelialization, fibroblast appearance, inflammation, collagenization, formation of vessel and skin appendages, and granulation tissue appearance were calculated using Masson's trichrome staining and H&E staining. The average of the scores obtained from two doctors of medicine (M.D.) and one veterinarian (DVM) were calculated for each histologic specimen. In addition, each analyst scored ten random histological tissues twice in a blinded manner, allowing for statistic intraobserver variability assessment.

2.7. Western blot

The presence of interleukin (IL)-6 in the skin specimen from each experimental group was examined using a Western blot. Western blotting was conducted based on the previously demonstrated methods [24]. The proteins of skin tissues were extracted using RIPA (radio immunoprecipitation assay) buffer and protease inhibitor (Sigma). The skin tissue lysate of each group was incubated on ice for 30min and then centrifuged at 13,000rpm for 15min at 4°C. The protein concentrations were quantified using bicinchoninic acid assay according to the manufacturer's instructions. The proteins were separated through 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred and blotted onto the nitrocellulose membrane. Nonspecific binding sites on the membrane were blocked in 5% skim milk powder in Tris Buffered Saline with 0.05% Tween 20 for 1h at room temperature. The membrane was then incubated with primary antimouse IL-6 or alpha-tubulin antibodies (Santa Cruz, CA, USA) overnight at 4°C. The ratio of IL-6 protein level to that in control was obtained using semiquantitative intensity analysis (normalized by the respective α-tubulin and background) by using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.8. Assessment of collagen production in vivo

Histological sections were stained with Masson's Trichrome to assess collagen content in rat skin after third-degree burns

with or without treatments. ImageJ software was used to determine the collagen content in each sample. In brief, wound samples from the control and experimental groups were stained and compared. The color settings in the ImageJ software was maintained at all times between the calculation of the blue-stained areas in samples. Magnification ×40 was employed to evaluate the samples and the calculation was repeated in four microscopic fields [25].

2.9. Statistical analysis

Independent experiments were repeated three times, and results were pooled from the repeated experiments. Data are presented as mean±standard error of the pooled data. Analysis of variance (ANOVA) and the Tukey–Kramer test were performed on GraphPad InStat 3 Software to determine significant differences ($P<0.05$) between experimental groups.

3. Results

3.1. TEM observation of farnesol-loaded liposomes

The morphology of farnesol-loaded liposomes was analyzed through TEM imaging. Farnesol-loaded liposomes, fabricated from DSPC, cholesterol, and farnesol, exhibited a spherical shape with a size ranged from approximately 27.2nm to 262nm. The majority of fabricated liposomes was about 137nm (42.6%). The TEM images and size distribution of farnesol-loaded liposomes are shown in Fig. 1. The encapsulation efficiency of the fabricated liposome-encapsulated farnesol was approximately 50%. A slow and gradual release profile of the fabricated farnesol-loaded liposomes was observed. Farnesol release rate from the prepared farnesol-loaded liposomes reached 35% within 168h (Fig. 1C).

3.2. Cytotoxicity of liposome-encapsulated farnesol on fibroblasts

In our previously published results showed that pure farensol concentration less than 0.4mM did not present any inhibition in fibroblasts viability. However, incubation with 0.8mM farnesol for 12h significantly reduced viability by approximately 90%, indicating cytotoxicity to fibroblasts in vitro. The effects of liposome-encapsulated farnesol on fibroblast proliferation in thist study were assessed using CCK-8 assay. As a result, treatment with liposome-encapsulated farnesol at the low concentration (0.04mM) or high concentration (0.8mM) did not lead to significant inhibition of L929 fibroblast proliferation (Fig. 2A), suggesting that liposome-encapsulation is capable of reducing toxicity of farnesol.

3.3. Effects of farnesol on collagen production in vitro

The effects of pure and liposomal farnesol on collagen formation by L929 fibroblasts were quantified using Sircol Soluble Collagen Assay. After treatment for 48h, pure farnesol at 0.1, 0.2 and 0.4mM significantly increased collagen production by skin fibroblasts, and 48h exposure of liposomal farnesol at 0.1–0.8mM enhanced the collagen production by the cells (Fig. 2 B and C). Exposure of pure farnesol at 0.4mM for 24h to normal skin fibroblasts promoted collagen production. The collagen production was increased by around 1.5–2.6 fold after exposure to 0.04–0.8mM liposomal farnesol for 24h while treatment with liposomal farensol from 0.2 to 0.4 mN for 48h particularly increased the collagen production by approximately 2.4–2.7-fold (Fig. 2C). The results demonstrated that liposomal encapsulation of farnesol and the gradual release of the compound from farnesol-loaded liposomes are beneficial for collagen production in L929 fibroblasts.

3.4. Determination of third-degree burns on the rat model

Third-degree burns of the untreated group were determined by measuring the burn depth and percentage of dermis burned through H&E staining. The average burn depth was 1962±55 μm, and the percentage of dermis burned was 98 ±1.5% on day 7 in the untreated rats (N=3). Namely, the depth of burns on day 7 post wounding in the untreated group reached nearly 2000 μm, in which the epidermal, dermal, and subcutaneous tissues was involved. Moreover, the percentage of dermis that was burned reached more than 95%. These data were in accordance with previous findings, indicating that third-degree burns were successfully established on the rat model [26].

3.5. Evaluation of the reparative effects of liposomal farnesol on third-degree burns in vivo

In the normal untreated group, rat skin subjected to both H&E and Masson's Trichrome staining showed intact structure of the epidermis, dermis, and subcutaneous layer on days 7 and 14 post burn (Figs. 3A–6A). In Fig. 3B, severe damage of full-thickness (third-degree) burns was created using heated

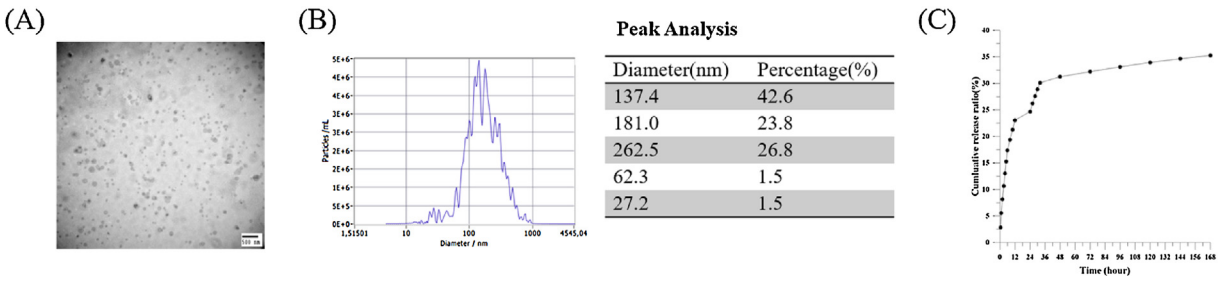

Fig. 1 – (A) The TEM images. (B) the size distribution of the fabricated farnesol-loaded liposomes and (C) In vitro drug release profiles of the prepared farnesol-loaded liposomes.

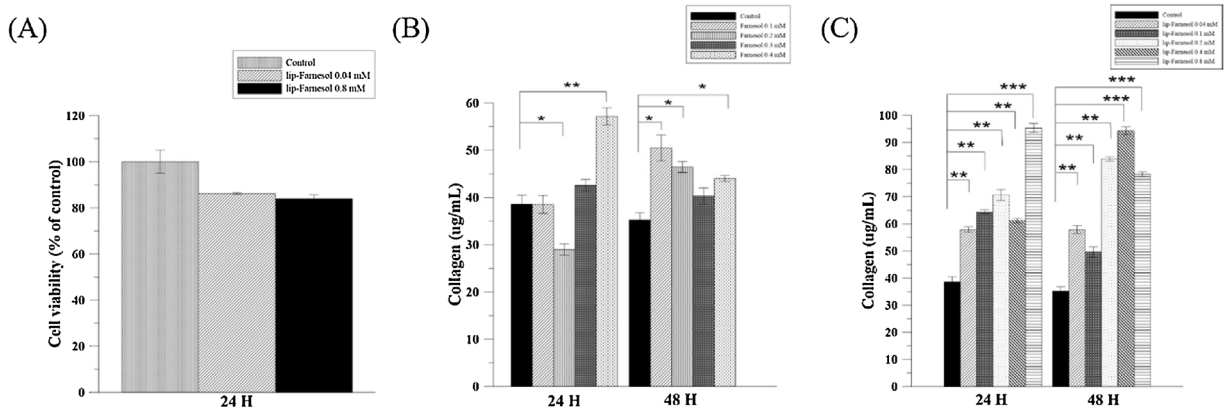

Fig. 2 – (A). Effects of liposome-encapsulated farnesol on normal skin fibroblast proliferation. (B) Stimulatory effects of pure and (B) liposomal farnesol on collagen production in vitro.

soldering iron on the rat skin. The injury extended from the epidermis through the dermis to the subcutaneous layer. No surviving follicle cells were observed on day 0 after creation of the full-thickness burns. The observations were consistent with those in Masson's Trichrome staining (Fig. 5B). The light red and pink staining referred to copious exudates and cytoplasm, resulting from extensive cell necrosis and death through the dermis to the subcutaneous tissue. On day 7 and day 14 after burns, histopathological observations showed that the prepared gel with a liposomal farnesol:HPMC ratios of 2:1 (group C, Figs. 3D and 5D) and 1:2 (group D, Figs. 4D and 6D) exerted the greatest effects on alleviating inflammation, enhancing collagen production and repairing skin tissues after third degree burns compared with the other experimental groups. The groups treated with the commercial SSD cream exhibited moderate skin tissue-restorative and collagen synthesis-increasing activities but less inflammation-easing effects on days 7 and 14 post burns than those exerted by our liposomal farnesol gel groups (Figs. 3F, 4E, 5F, and 6E). The HPMC alone gel did not show clear wound healing-improving effects on days 7 and 14 post burns (Figs. 3G, 4F, 5G, and 6F).

3.6. *Histopathological scoring of wound healing post wounding*

The healing scores of each group on day 7 and 14 post burns are respectively presented in Tables 1 and 2. On day 7 post wounding, the group treated with a liposomal farnesol:HPMC ratio of 2:1 had the highest healing scores (18.33±0.33) among the experimental groups, followed by the group treated with a liposomal farnesol:HPMC ratio of 1:2 (15.33±0.67) and that treated with the commercial SSD cream (13.33±0.67). The untreated group (5.00±0.58) and HPMC alone group (7.33 ±0.33) presented significant lower healing scores. On day 14 afterburns, the group treated with a liposomal farnesol: HPMC ratio of 1:2 achieved the highest healing scores (22±0 .58), and the groups treated with a liposomal farnesol:HPMC ratio of 2:1 (19.33±0.33) and commercial SSD cream (15.00 ±0.58) exhibited significantly higher scores than the untreated (9.33±1.20) and HPMC alone groups (7.33±0.88). The wound healing scoring results were consistent with the histopathological observations and in vivo collagen-production quantitative data.

3.7. *Stimulatory effects of liposomal farnesol gel on collagen production in vivo*

On day 7 post wounding, the prepared liposomal farnesol gel (2:1) exerted the greatest collagen production-increasing effects compared with the untreated or commercial SSD cream-treated groups. The prepared liposomal gel (1:2) also exhibited significantly greater collagen synthesis-enhancing effects on day 7 post wounding compared with the group without treatment (Fig. 6G). On day 14 post wounding, the prepared liposomal farnesol gel (1:2) presented the strongest collagen production-promoting activity compared with the untreated group and the groups treated with commercial SSD cream or liposomal farnesol gel (2:1).

3.8. *Determination of IL-6 in vivo through Western blotting*

The presence of IL-6 proteins on day 7 and 14 post wounding in the experimental groups in vivo was determined through Western blotting (Fig. 7). On day 7 post wounding, the group treated with the liposomal farnesol:HPMC=2:1 gel showed the lowest IL-6 content compared with the other experimental groups. This result was consistent with the histopathological findings on day 7 after burns, in which our prepared gel (2:1) exhibited the highest inflammation-inhibitory effects among the experimental groups (Figs. 3D and 5D). On day 14 post wounding, however, the group treated with the prepared gel (liposomal farnesol:HPMC=1:2) exhibited the lowest IL-6 content among the groups. The results of the IL-6 Western blot on day 14 post wounding were also predominantly consistent with the histopathological observations on day 14 after burns, in which the minimal inflammatory exudates were found compared with the other experimental groups (Figs. 4(D) and 6(D)). In addition, the commercial SSD cream did not reduce the amount of IL-6 proteins on day 7 or day 14 post wounding and barely exerted inflammation-attenuating effects in histopathological findings in vivo (Figs. 3F, 5F, 4E and 6E).

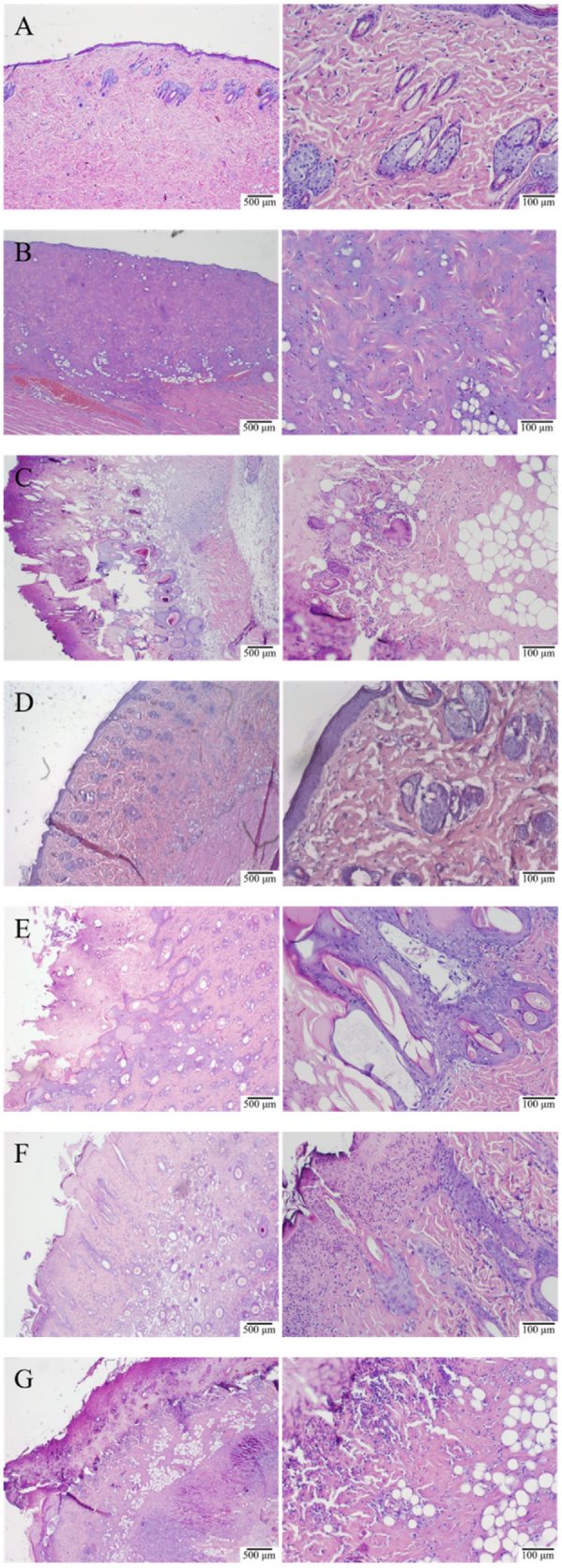

Fig. 3 – H&E staining of the rat skin after third-degree burns with or without treatments on day 7 postwounding. (A): normal rat skin. (B): the rat skin on day 1 postburns without treatments. (C): the rat skin on day 7 postburns without treatments. (D): the rat skin on day 7 postburns treated with the gel of liposomal farnesol: HPMC (2:1). (E): the rat skin on day 7 postburns treated with the gel of liposomal farnesol: HPMC (1:2). (F): the rat skin on day 7 postburns treated with commercial SSD cream. (G): the rat skin on day 7 postburns treated with the gel of HPMC alone.

4. Discussion

The most commonly used burn models are those of the contact burn and the scalding burn [27]. Previous studies have found that farnesol exhibits antibacterial activities against important pathogens of skin infections, such as *Staphylococcus aureus* [28]. Furthermore, the antioxidant and inflammation-alleviating effects of farnesol have been demonstrated in several studies [11,20,29]. In the current study, treatment with liposomal farnesol-containing gel yielded obvious wound healing-accelerating action in vivo in rats with third-degree burns. Our current in vitro data revealed that treatment with liposomal farnesol exhibited strong and significant collagen production-enhancing effects in skin fibroblasts compared with control. These in vitro data suggest that liposomal farnesol, because of its sustained release and reduced cytotoxicity activities, may exert improved wound healing effects than pure farnesol in vivo. These findings show clear effects of liposomal farnesol on improving wound healing and increasing collagen synthesis post burn in a rat model and confirm previous in vitro findings. On day 7, limited repair of the dermis was found without treatment in the group that suffered full-thickness burns (group B, Figs. 3C and 5C), as evident by the limited amount of collagen and hair follicles, compared with that in the groups that were treated with liposomal farnesol-containing HPMC or commercial gel (groups C, D and E, Figs. 3D/5D, 3E/5E and 3F/5F). Furthermore, the extent of restorative effects on third-degree burns was observed among the different treated groups (groups C–F) on day 7. Liposomal farnesol containing HPMC (Group C) exerted the greatest therapeutic effects on third-degree burns at day 7, evident by the large amount of collagen production, enhanced numbers of hair follicles in the dermis, and minimal exudates in the epidermis as well as evidently reduced swollen capillaries (Figs. 3D and 5D) compared with the other groups. The histopathological findings were consistent with IL-6 Western blot, collagen-production quantitative data, and postburn wound healing scores, in which liposomal farnesol gel (2:1) exerted the strongest semiquantitative effects of easing inflammation, promoting collagen production, and improving total wound healing scores among the experimental groups (Table 1). These in vivo findings indicated that a 7-day treatment regimen with liposomal farnesol gel containing HPMC (2:1) after three-degree burns exhibited the greatest actions on acceleration of wound healing and attenuation of inflammation. The histopathological observations were consistent with the in vivo results of the collagen-production quantitative assay, IL-6 Western blot, and total wound healing scores. The other prepared liposomal farnesol with HPMC (liposomal farnesol:HPMC=1:2) showed similar actions on collagen production but less stronger effects on hair follicle formation in the dermis, coupled with the thicken epidermis

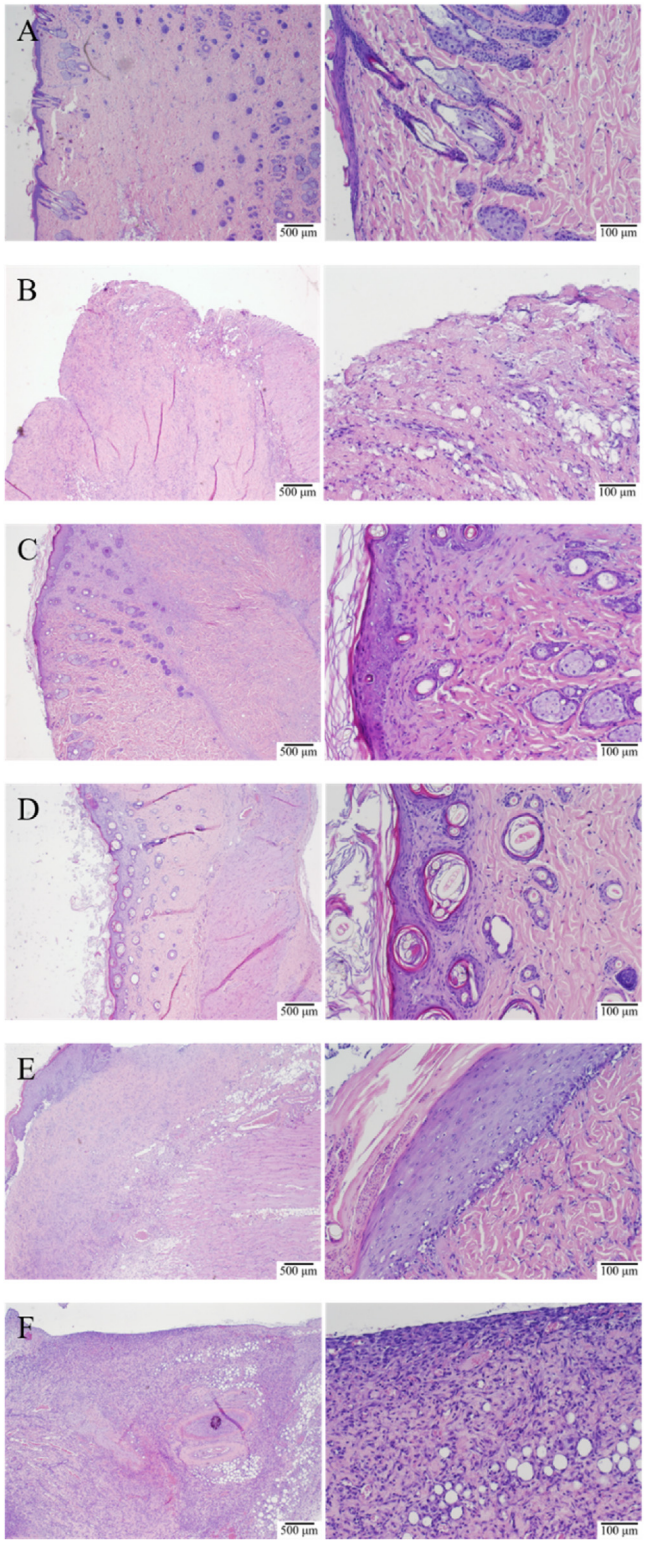

Fig. 4 – H&E staining of the rat skin after third-degree burns with or without treatments on day 14 postwounding. (A): normal rat skin. (B): the rat skin on day 14 postburns without treatments. (C): the rat skin on day 14 postburns treated with the gel of liposomal farnesol: HPMC (2:1). (D): the rat skin on day 14 postburns treated with the gel of liposomal farnesol: HPMC (1:2). (E): the rat skin on day 14 postburns treated with commercial SSD cream. (F): the rat skin on day 14 postburns treated with the gel of HPMC alone.

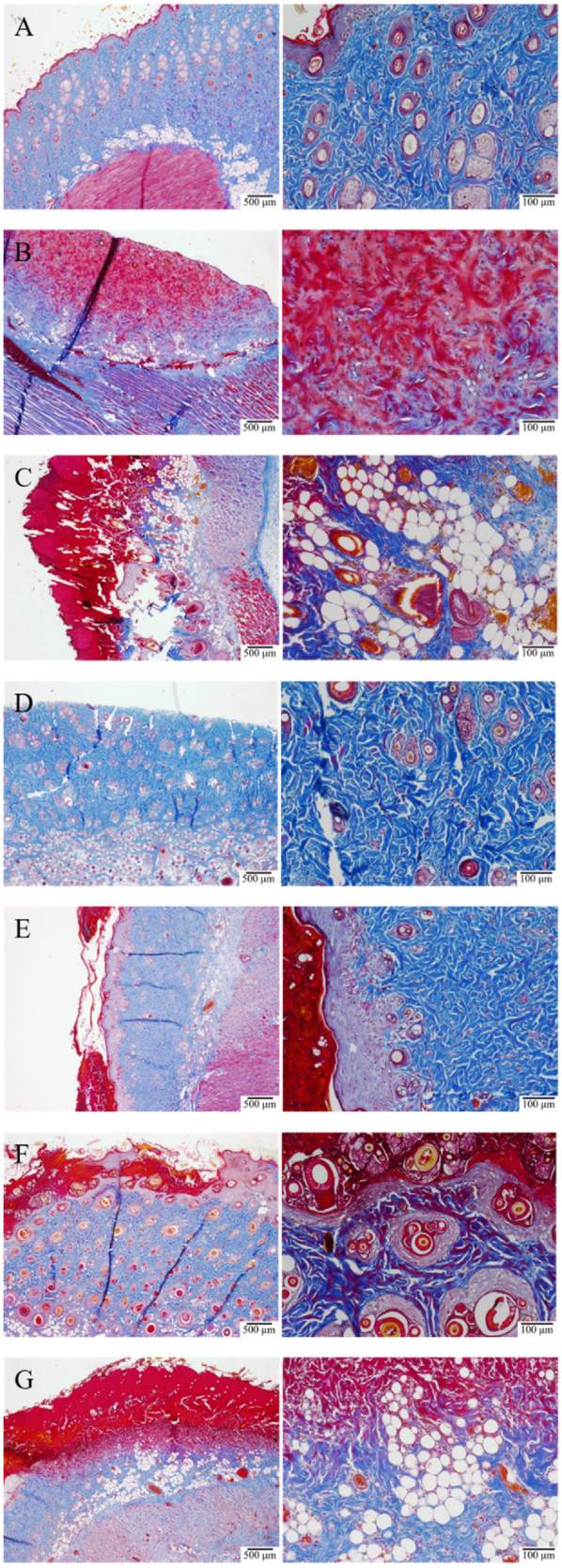

Fig. 5 – Masson's Trichrome staining of the rat skin after third-degree burns with or without treatments on day 7 postwounding. (A): normal rat skin. (B): the rat skin on day 1 postburns without treatments. (C): the rat skin on day 7 postburns without treatments. (D): the rat skin on day 7 postburns treated with the gel of liposomal farnesol: HPMC (2:1). (E): the rat skin on day 7 postburns treated with the gel of liposomal farnesol: HPMC (1:2). (F): the rat skin on day 7 postburns treated with commercial SSD cream. (G): the rat skin on day 7 postburns treated with the gel of HPMC alone.

Table 1 – The wound healing scores in each experimental group on day 7 after third-degree burns.

Group	A	B	C	D	E
Epithelialization (0–4)	0.67±0.33	2.33±0.33[a*]	2.67±0.33[a*]	2.67±0.33[a*]	1.33±0.33
Fibroblast appearance (0–4)	1.33±0.33	2.67±0.33	2.67±0.33	2.67±0.33	2.67±0.33
Inflammation (0–4)	0.33±0.33	0.67±0.33	3.00±0.00 [a***,b**,c**,d*]	1.33±0.33	0.67±0.33
Formation of vessel and skin appendages (0–4)	1.00±0.00	2.67±0.33[a**,c**]	3.33±0.33 [a***,c***]	2.67±0.33 [a**,c**]	1.00±0.00
Collagenization (0–4)	1.00±0.00	2.33±0.33[a**,c**]	3.33±0.33 [a***,b*,c***]	3.00±0.00 [a***,c***]	1.00±0.00
Granulation tissue (0–4)	0.67±0.33	2.67±0.33[a**,c**]	3.33±0.33[a***,c***]	3.00±0.00[a**,c**]	0.67±0.33
Total (24)	5.00±0.58	13.33±0.67[a***,c***]	18.33±0.33[a***,b***,c***,d*]	15.33±0.67[a***,c***]	7.33±0.33

Table 2 – The wound healing scores in each experimental group on day 14 after third-degree burns.

Group	A	B	C	D	E
Epithelialization (0–4)	1.00±0.00	2.67±0.33[a**,c***]	3.00±0.00[a**,c***]	3.33±0.33[a***,c***]	0.33±0.33
Fibroblast appearance (0–4)	2.67±0.33	3.33±0.33	3.67±0.33	3.67±0.33	3.00±0.00
Inflammation (0–4)	1.67±0.33	1.33±0.33	2.33±0.33[c*]	3.67±0.33[a**,b**,c***,d*]	0.67±0.33
Formation of vessel and skin appendages (0–4)	1.33±0.33	2.33±0.33	3.67±0.33[a**,c**]	3.67±0.33[a**,c**]	1.33±0.33
Collagenization (0–4)	1.67±0.33	2.33±0.33	2.67±0.33	4.00±0.00[a**,b*,c**]	1.33±0.33
Granulation tissue (0–4)	1.00±0.00	3.00±0.00[a**,c***]	3.67±0.33[a***,c***]	3.67±0.33[a***,c***,d*]	0.67±0.33
Total (24)	9.33±1.20	15.00±0.58[a**,c**]	19.33±0.33[a***,b*,c***]	22±0.58[a***,b***,c***]	7.33±0.88

Inflammation: Severe (0) Moderate (1) Mild (2) mild-none (3) None (4).
Scores were expressed as Mean±SEM, significant differences were determined by ANOVA with Tukey–Kramer test.
A: untreated group, B: commercial group, C: 2:1 group, D: 1:2 group, E: HPMC alone group.
[a] *P<0.05, **P<0.01, ***P<0.001, compared to untreated group.
[b] *P<0.05, **P<0.01, ***P<0.001, compared to commercial group.
[c] *P<0.05, **P<0.01, *** P<0.001, compared to HPMC alone group.
[d] *P<0.05, compared between 2:1 and 1:2 liposomal farnesol gel groups.

containing exudates but less swollen capillaries (group D, Figs. 3E and 5E), suggesting that this prepared formula (1:2) also possessed beneficial effects on third-degree burns but lower inflammation-easing effects compared with group C. Consistently, identical results were also found in the postburn healing scores (Table 1). The inflammatory (early) phase after burns is crucial for not only preventing infection during healing but also destroying necrotic tissue and provide signals necessary for tissue repair [30]. These signals are key factors to recruit leukocytes and macrophages that initiate the proliferative phase [27]; however, excessive or sustained edema, hypermetabolism, and inflammation can aggravate pain, delay re-epithelialization, and thereby worsen wound healing [31,32]. Because it has been previously shown that the level of inflammation and hypermetabolism is associated with the extent and depth of burn [33–35], the appropriate control of inflammation is essential for healing, particularly deeper wounds such as third-degree burns. After the inflammatory phase after severe burns, alleviation of excessive inflammation is helpful for wound healing. Studies have shown that effective attenuation of inflammation exerted by Chinese medicine or herbal extracts can facilitate healing of burn wounds. We previously demonstrated that the plural farnesol gel reduces inflammation in UVB-induced sunburns. Therefore, this finding is reasonable considering that group C contained twofold higher amount of liposomal farnesol than group D, suggesting that group C can exhibit stronger inflammation-reducing actions than group D. This was also in accordance with the largely reduced IL-6 content, as demonstrated by the Western blot (Fig. 7). In the group treated with topical 1% SSD and 0.2% chlorhexidine digluconate (Silvazine), or group E (Figs. 3F and 5F), lower collagen production and hair follicle formation, together with a thickened epidermis and clear infiltration of inflammatory cells, were found on day 7 after the cream was administered, showing fewer inflammation-inhibitory and collagen formation-stimulating effects compared with group C. These findings demonstrated that 1% SSD and 0.2% chlorhexidine digluconate have limited effects on attenuation of inflammation and promotion of collagen formation at day 7 after topical administration, compared with those found in liposomal farnesol plus HPMC (2:1) treated group (group C). Therefore, the inflammation-inhibitory and collagen formation-promoting effects of our prepared gel containing liposomal farnesol are superior to those of the commercial cream for burns. In the rat model, the groups that were given the gel with HPMC but without liposomal farnesol (group F, Figs. 3G and 5G) demonstrated similar signs (injured and swollen epidermis, severely damaged dermis and subcutaneous tissues, and clear inflammatory cell infiltration) as the rats that received no treatment (group B). These results indicate that liposomal farnesol is the major component that exerts reparative effects on third-degree burns and HPMC itself did not attenuate inflammation or promote wound healing of full-thickness burns.

At day 14 post burn, severe damage to the epidermis and dermis remained in the rat skin in the no treatment group (group B, Figs. 4B and 6B) and in the group treated with the HPMC gel but without liposomal farnesol (group F, Figs. 4F and 6F). These findings were consistent with those observed at day

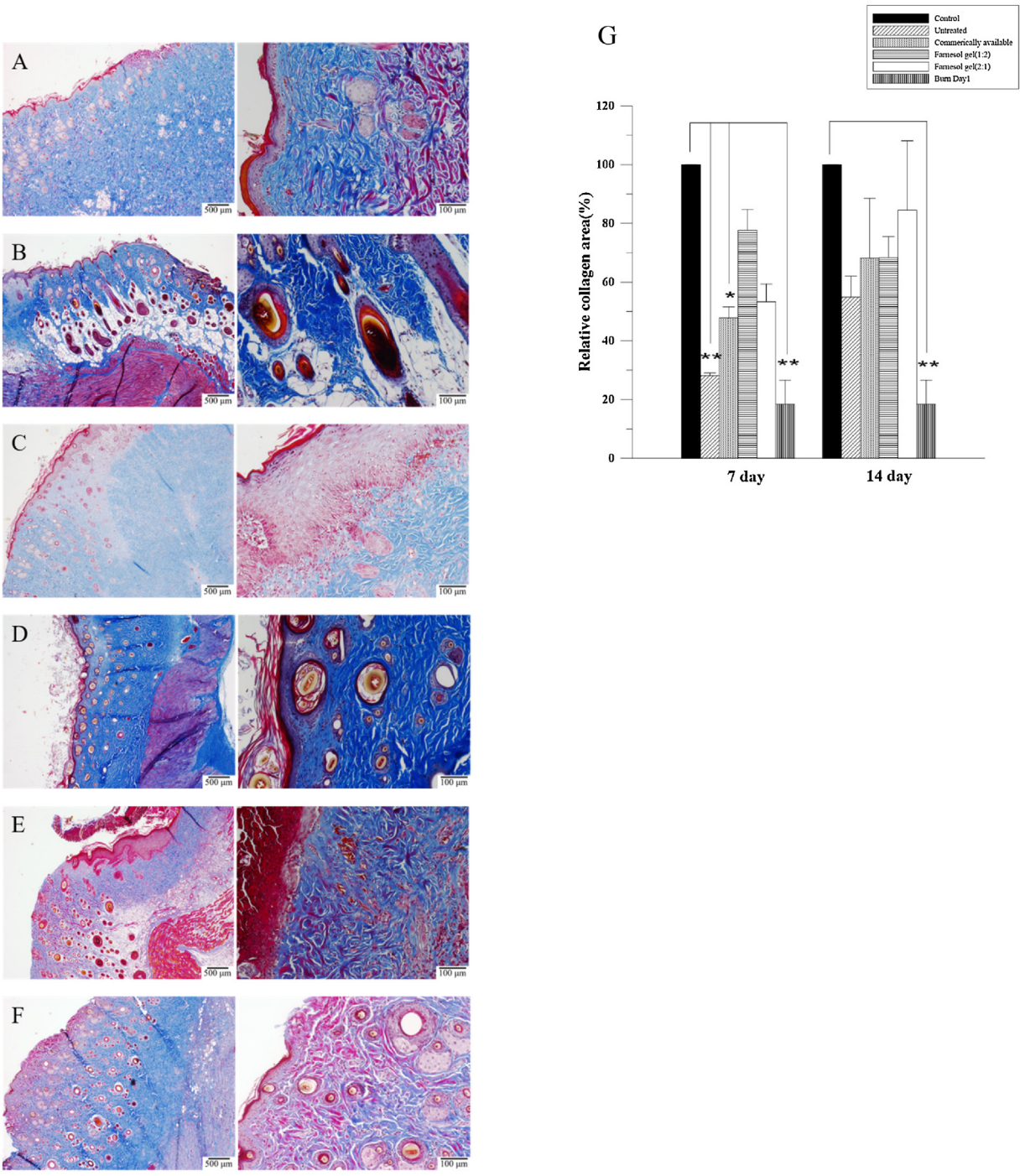

Fig. 6 – Masson's Trichrome staining of the rat skin after third-degree burns with or without treatments on day 14 postwounding. (A): normal rat skin. (B): the rat skin on day 14 postburns without treatments. (C): the rat skin on day 14 postburns treated with the gel of liposomal farnesol: HPMC (2:1). (D): the rat skin on day 14 postburns treated with the gel of liposomal farnesol: HPMC (1:2). (E): the rat skin on day 14 postburns treated with commercial SSD cream. (F): the rat skin on day 14 postburns treated with the gel of HPMC alone. (G) Effects of liposomal farnesol gel on collagen production on day 7 (A) and day 14 (B) after burns in vivo.

7 in these groups. However, the wounds in groups C (Figs. 4C and 6C) and D (Figs. 4D and 6D), which received our prepared liposomal farnesol:HPMC gels, showed obvious reparations in the epidermis and dermis, demonstrated by the more intact epidermis, collagen in the dermis, and hair follicle formation in groups C and D. After 14 days of treatment, group D (Figs. 4D and 6D) showed greater effects on collagen production and hair follicle formation in the dermis and less inflammatory exudates in the epidermis than those in group C (Figs. 4C and 6C) and all the other experimental groups. These results

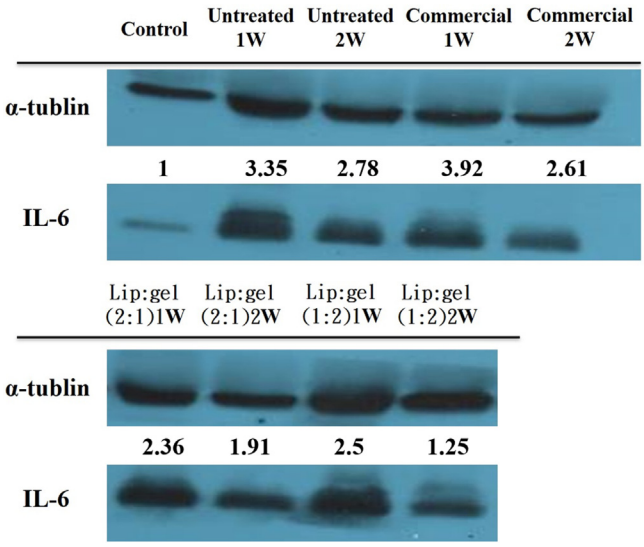

Fig. 7 – Determination of IL-6 by Western blot in each experimental group.

suggest that at stage 2 of wound healing, the presence of farnesol is essential, but is required in little amounts for most appropriate tissue repair of post-third-degree burns in the rat skin. These histopathological observations were also in accordance with the data of in vivo IL-6 Western blot, collagen production assessment, and healing scores on day 14 post burn, in which liposomal farnesol:HPMC=1:2 also exhibited the greatest collagen synthesis activity and had the highest healing scores compared with the other experimental groups (Fig. 6G, Fig. 7, and Table 2). This is interesting because we found that the concentrations of pure farnesol over 0.3mM exerted significant cytotoxic effects on normal skin fibroblast viability in vitro [20]. In the group treated with liposomal farnesol:HPMC at 2:1, fibroblasts after third-degree burns may be exposed to the accumulated amount of farnesol in vivo, resulting in decreased collagen production by skin fibroblasts, despite the slow and sustained release characterized by the liposomes. Skin fibroblasts play major roles in the second (proliferative) and third (remodeling) phases of wound healing in the skin. These include the synthesis and secretion of ECM, such as collagen, fibronectin, proteoglycans, and glycosaminoglycans, and maturation of wound scar by the deposition of collagen and elastin [36,37]. The second phase commences on the third day after wounding and continues for approximately 2 weeks afterward [37]; therefore, the effects of topical administration of our prepared therapies (liposomal farnesol:HPMC=2:1 or 1:2) were assessed for 14days during the proliferative phase. Therefore, despite exerting collagen production-increasing effects in vitro, a high concentration of farnesol could inhibit cell viability on skin fibroblasts in vivo at day 14 after the liposomal farnesol-containing gel has been administered. This could possibly decelerate wound healing on day 14 post burns.

Regarding the group that received treatment with a combination of 1% commercial SSD and 0.2% chlorhexidine digluconate for 14 days, the formation of hair follicles was quite limited, and a clear inflammatory exudative layer was observed beneath the keratin of the epidermis. The restorative effects of this combination on the burns appeared more favorable than those in group B and group F but inferior to those in groups C and D.

The importance and correlation of cytokines with mortality and survival of patients after burn injury have been studied. IL-1 receptor antagonist (IL-1RA), IL-6, and monocyte chemoattractant protein-1 (MCP-1) have been shown to be prognostic indicators of mortality in burn patients [38]. Another study also demonstrated that IL-6 is one of the important mediators in inflammatory changes after a burn injury [39,40]. Notably, in severely burned rats, serum IL-6 was undetectable in sham-treated rats. IL-6 peaked during the early hours post burn and levels were proportionate to the size of the burn area in rats; IL-6 increased early on day 1 and a few days after burn injury [41]. It has also been found that only IL-6 is a sustained increasing mediator of burn-injury pain after burn injury in rats [42]. These reports indicated that IL-6 is a crucial and appropriate cytokine for evaluating inflammation, pain, severity, and survival after burn injury. The current data revealed that our prepared liposomal farnesol gel at ratios of 2:1 and 1:2 efficiently reduced IL-6 level on days 7 and 14 post burns. This indicates that our prepared liposomal farnesol gel not only alleviates the inflammation after severe burns but also reduces the pain scale and severity of injury post burns.

Taken together, the histopathological findings in the current work were coherent with the in vivo results of postburn wound healing scores, IL-6 Western blot, and collagen-production quantitative assay. These in vivo data demonstrate that liposomal farnesol gel at various concentrations can promote collagen production and improve tissue repair at different stages of wound healing after third-degree burns.

5. Conclusions

The prepared liposomal farnesol/HPMC gel in this study exhibited effective collagen production-enhancing and wound healing-improving effects in vitro and in vivo, despite

inhibition on fibroblast proliferation at high concentrations. The fabricated liposome-encapsulated farnesol gel possessed significantly greater effects on wound healing after third-degree burns compared with the untreated, HPMC gel alone, and commercial SSD cream treated groups. To attain promotion of wound healing and prevention of infection, a combined therapy of our prepared liposomal farnesol/HPMC gel with the antimicrobial cream/solutions may be an alternative for postburn treatment.

Declaration of interests

None.

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