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Wound healing effect of silk fibroin/alginate-blended sponge in full thickness skin defect of rat

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Abstract Silk fibroin (SF) and alginate (AA) have been proved to be invaluable natural materials in the field of biomedical engineering. This study was designed to compare the wound healing effect of SF, AA and SF/AA-blended sponge (SF/AA) with clinically used Nu Gauze^{TM} (CONT) in a rat full thickness wound model. Two circular skin wounds on the back of rat were covered with either of CONT, SF, AA or SF/AA. On the postoperative days of 3, 7, 10 and 14, residual wound area was calculated, and skin wound tissues were biopsied to measure the area of regenerated epithelium and collagen deposition as well as the number of proliferating cell nuclear antigen (PCNA)-immunoreactive cells. Half healing time (HT_{50}) of SF/AA was dramatically reduced as compared with that of SF, AA or CONT. Furthermore, SF/AA significantly increased the size of re-epithelialization and the number of PCNA positive cells, whereas the effect of SF/AA on collagen deposition was not significantly different as compared with that of SF or AA. These results demonstrate that the wound healing effect of SF/AA is the best among other treatments including SF and AA, and this synergic effect is mediated by re-epithelialization via rapid proliferation of epithelial cell.

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1. Introduction

A wound effectively represents a disruption of normal skin physiology and the healing processes the mechanism by which the body seeks to re-establish skin continuity [1]. A full thickness skin wound model is one of the most appropriate models that is used to evaluate the wound healing effect of various remedy application such as ointment, spray and bandage. This cutaneous excision wound is healed through several dynamic processes, including clot formation, granulation tissue proliferation, contraction and re-epithelialization [2]. The precise measurement of these processes (e.g. the size of re-epithelialization and collagen deposition) has been frequently utilized to analyze the effect of any treatments for degree of wound healing [3–5]. Furthermore, it has been reported that proliferating cell nuclear antigen (PCNA) expression is closely correlated with migration of keratinocytes and epithelial cell during wound healing [6–9].

The silk fibroin (SF) from the *Bombyx mori* is a structural polymer possessing unique physical properties including good biocompatible and easy structural transition form

random coil to β-sheet structure by mechanical or physical treatments. It has been established to be invaluable material in the field of biomedical engineering ranging from skin and vascular grafts to substrates for mammalian cell culture [10]. In the clinical treatment of skin defect, SF is known to be useful material that promotes collagen synthesis and re-epithelialization. Furthermore, SF was considered to be proper for the generation of biomedical products such as blended materials because of its minimal adverse effects on the immune system [3].

The alginate (AA) is an algal polysaccharide consisting mainly of beta-1,4-linked D-mannuronic acid and alpha-1, 4-linked L-glucuronic acid. When AA comes into contact with wound exudate, ion exchange occurs between the calcium ions of the AA and the sodium ions in the exudate resulting in the formation of a gel on the surface of the wound. This gel absorbs moisture and maintains an appropriately moist environment that is considered to promote optimal wound healing [11]. For these reasons, SF/AA-blended sponge is likely to be an effective material that can be used for wound dressing and provide the necessary requirements for recovery.

The aim of the present study is to compare the wound healing effect of SF, AA, and SF/AA-blended sponge with that of clinically used Nu Gauze™ in full thickness wound model of rats. Furthermore, it is to evaluate whether the wound healing effect of SF/AA-blended sponge is mediated by either re-epithelialization or granulation process using histological observation and immunohistochemical analysis for PCNA.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (weighing 200 to 250 g; Laboratory Animal Center of Seoul National University, Seoul, South Korea) were used in this experiment. The rats were kept in a colony room with an ambient temperature of 22°C and a 12 h alternating light-dark cycle (7:00 AM onset). Food and water were supplied *ad libitum*. All surgical and experimental procedures used in the present study were approved by the Animal Care and Use Committee at Seoul National University and conform to NIH guidelines (NIH publication No. 86-23, revised 1985).

2.2. Full thickness wound model

Rats were anesthetized using an intraperitoneal injection of 4% chloral hydrate (1 ml/100 g body weight), and the dorsal hair of the rat was shaved with a clipper, and disinfected with 70% ethanol and betadine. Two full-thickness wound of 2 cm diameter was marked using a template and the tissue excised to the level of the panniculus carnosus using dissecting scissors and forceps. All rats were intraperitoneally pretreated with antibiotics (streptomycin sulfate, Chong Kun Dang, Seoul, South Korea) 2 hr prior to skin excision.

2.3. Preparation of silk fibroin/alginic acid-blended sponge

SF- and AA-sponges as well as SF/AA-blended sponges were prepared by the same method previously reported [12]. Briefly, SF-, AA-sponges and SF/AA-blended sponges were prepared by mixing 1 (w/v)% of aqueous SF and 1 (w/v)% of AA solution. Each solution was blended with weight ratios of SF to AA to be 10:0, 5:5 and 0:10 and stirred at room temperature for 30 min. The blend solution was cast on polystyrene dishes and freeze-dried to obtain test materials.

2.4. Experimental group

All sponges were cut off into the same size with skin wound area and subsequently each skin wound of rats was softly covered with silk fibroin sponge (SF, $n = 8$), alginate sponge (AA, $n = 8$) or silk fibroin/alginate-blended sponge (SF/AA, $n = 8$), respectively. Clinically used Nu Gauze™ (Johnson & Johnson Co., USA) treatment was applied for control group (CONT, $n = 8$). After each sponge treatment, the sterilized elastic bandage (Tegaderm™ and Coban™, 3M Health Care, USA) was used for the prevention of sponge omission.

2.5. Measurement of residual wound area and half healing time (HT_{50})

In order to determine the wound-healing curve and half healing time (HT_{50}), the residual wound margin was drawn with tracing film at 0, 3, 7, 10, 14 days after skin excision. The labeled film scanned and the wound area was calculated by image analysis software (Metamorph, Universal Imaging Co., USA). Rate of wound healing was expressed as the percentage of remained area. Residual Wound Area (%) = $[R_{(3,7,10,14)}/R_{(0)}] \times 100$, where $R_{(0)}$ and $R_{(3,7,10,14)}$ represent the remained area at postoperative days 0 and 3, 7, 10, 14, respectively. The wound healing curve was gained by the Boltzman equation for fitting and the value of HT_{50} was calculated.

2.6. Histological and immunohistochemistry procedures

2.6.1. Tissue preparation

This study was performed in separated group [SF ($n = 6$), AA ($n = 6$), SF/AA ($n = 6$) and CONT group ($n = 6$) at each time point (3, 7, 10, 14 days)], but all experimental procedures were same as previous wound closure measurement. On the postoperative 3, 7, 10, 14 days, skin tissues were harvested from the central regions of the wound by 0.5 cm × 2.5 cm strip and fixed in 10% neutral buffered formalin (NBF) and embedded in paraffin. Sections of 5 μm thickness were cut according to routine procedures, mounted on poly-lysin coated slides. Sections were incubated 43°C for 4 h, deparaffinized in xylene and hydrated in decrease concentration of ethanol.

2.6.2. Measurement of re-epithelialization and collagen deposition

At four tissue sections per sample strip, Hematoxylin-Eosin staining and Masson's trichrome staining were performed to measure the area of regenerated epithelium and collagen deposition at wound margins, respectively. Each stained slide was placed on the stage of the microscope, where the image was then displayed on a monitor screen via CCD camera (Micromax Kodak1317, Princeton Instruments, USA) connected to a computer-assisted image analysis system (Metamorph, Universal Imaging Co., USA). The area of regenerated epithelium was measured from each wound margin side at 500 μm length. The area of collagen deposition at the dermis was measured in the three regions at both ends and middle with 300 × 300 μm^2 rectangular area.

2.6.3. Measurement of proliferating cell nuclear antigen (PCNA)-immunoreactive cells

At four tissue sections per skim sample, proliferative cells in wound area were identified using a monoclonal antibody to proliferating cell nuclear antigen (PCNA). The deparaffinized section was incubated with 0.3% hydrogen peroxide in PBS and preblocking with 1% normal rabbit serum and 0.3% triton X-100 in PBS. Subsequently, sections were incubated in monoclonal mouse anti-PCNA antibody (1:100, DAKO, USA) at 4°C overnight and the sections were incubated in biotinylated rabbit anti-mouse IgG (1:200, Vector Labs, USA) for 1 h at room temperature. Following several washes, the tissue sections were processed with the avidin-biotin (ABC) method (ABC kit, Vector Labs, USA). Finally, PCNA immunoreactive neurons were visualized using a 3,3′-diamino-benzidine reaction with 0.2% nickel chloride intensification (yielding black labeled neuronal nuclei).

The quantitative image analysis of PCNA-immunoreactive cells was generally similar as the measurement of re-epithelialization and collagen deposition as above described. In order to maintain constant threshold for each image and to compensate for subtle variability of immunostaining, we only counted neurons that were at least 70% darker than average gray level of each image after background subtraction and shading correction were performed. The number of PCNA-positive epidermal cell was counted in both margin areas (300 × 300 μm^2 dimension), and mean value was calculated.

2.7. Statistics

All values were expressed as the mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was performed to determine the overall drug effect. Student Newman-Keuls test was subsequently performed to check for differences among experimental groups. Throughout the analyses, P value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Effect of silk fibroin-alginate (SF/AA)-blended sponges on the degree of wound healing

The wound size was significantly reduced from 7 days after treatment in the SF, AA and SF/AA groups as compared with that of Nu Gauze^{TM}-treated control group (CONT) (Fig. 1A, $^{*}p < 0.05, ^{**}p < 0.01$). Moreover, the wound size of SF/AA group was significantly decreased as compared with either those of SF or AA group (Fig. 1A, $^{+}p < 0.05, ^{\#}p < 0.05$ as compared with SA or SF, respectively).

Half healing time (HT_{50}) was compared in each groups (Fig. 1B). Not only SF/AA sponge treatment but also SF or AA sponge treatment significantly decreased HT_{50} as compared with that of control group (CONT) ($^{**}p < 0.01$). Furthermore, there was significant reduction of HT_{50} in SF/AA group as comparison to either SF group or AA group (Fig. 1B, $^{+}p < 0.05, ^{\#}p < 0.05$ as compared with SA or SF, respectively).

3.2. Effect of silk fibroin-alginate (SF/AA)-blended sponge on the re-epithelialization and collagen deposition

The area of new epithelialization tissue was markedly increased from 7 days after treatment in SF, AA and SF/AA

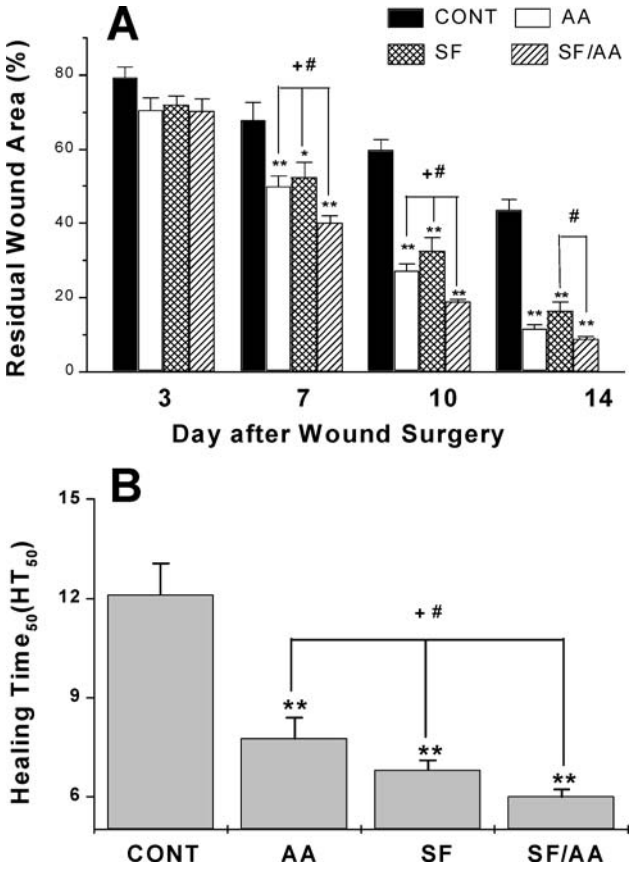

Fig. 1 The effect of silk fibroin (SF), alginic acid (AA) and silk fibroin-alginic acid (SF/AA)-blended sponges on the residual wound area (A) and on half healing time (HT_{50}) (B). Each bar represented mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with Nu Gauze^{TM}-treated group (CONT), $^{+}p < 0.05$ compared with AA and $^{\#}p < 0.05$ compared with SF.

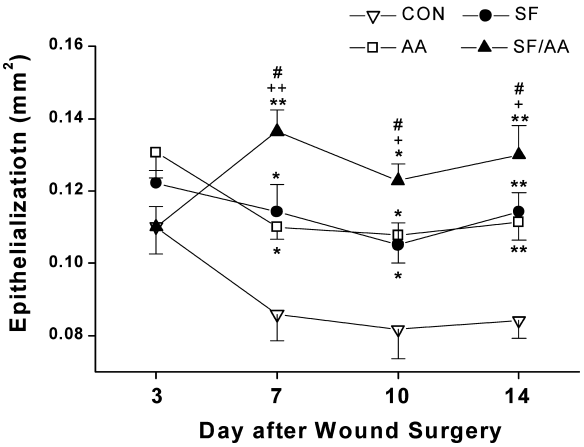

Fig. 2 Graph showing the proliferative effect of silk fibroin (SF), alginate (AA) and silk fibroin-alginate (SF/AA)-blended sponges on the re-epithelialization of the full-thickness excision. Each value represented mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with Nu Gauze^{TM}-treated group (CONT), $^{+}p < 0.05$, $^{++}p < 0.01$ compared with AA and $^{\#}p < 0.05$ compared with SF.

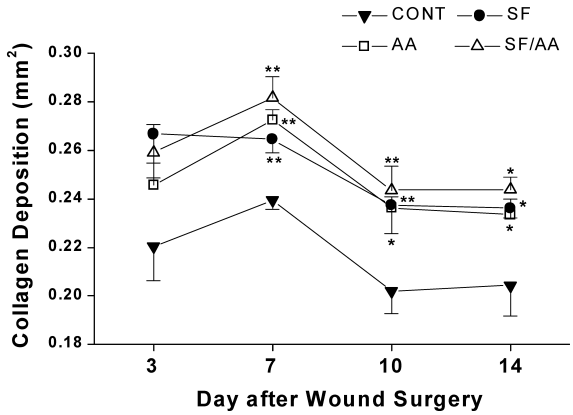

Fig. 3 Graph showing the proliferative effect of silk fibroin (SF), alginate (AA) and silk fibroin-alginate (SF/AA)-blended sponges on the collagen deposition of the full-thickness excision. Values represented mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with Nu Gauze^{TM}-treated group (CONT).

sponges-treated groups as compared with those in CONT (Fig. 2, $^{*}p < 0.05$, $^{**}p < 0.01$). In addition, SF/AA-treated group produced a significant effect on re-epithelialization of wound as compared with AA- and SF-treated group (Fig. 2, $^{+}p < 0.05$, $^{++}p < 0.01$ as compared with AA and $^{\#}p < 0.05$ as compared with SF).

In addition, the area of collagen deposition of granulation tissue was significantly increased from 7 days after treatment in SF, AA and SF/AA groups as compared with those in CONT (Fig. 3, $^{*}p < 0.05$, $^{**}p < 0.01$). However, there was no difference among SF, AA and SF/AA groups.

3.3. Effect of silk fibroin-alginate (SF/AA)-blended sponge on proliferating cell nuclear antigen (PCNA)-immunoreactive cell

The value of PCNA positive cells was evaluated in SF, AA and SF/AA sponge-treated groups as well as CONT(Fig. 4). The number of PCNA positive cells was significantly increased from 3 days after treatment in SF and SF/AA groups and from 10 days in AA group as compared with those of CONT ($^{*}p < 0.05$, $^{**}p < 0.01$). Furthermore, there are more PCNA positive cells at 7 and 10 days after treatment in SF/AA group as compared with either that of AA or SF group (Fig. 4, $^{+}p < 0.05$, $^{\#}p < 0.05$ as compared with AA or SF, respectively). The immunohistochemical finding of PCNA-immunoreactive cells at 7 days after treatment was shown in Fig. 5. The PCNA-positive cells in epidermis were markedly increased in SF/AA group (Fig. 5B) as compared with those of CONT (Fig. 5A).

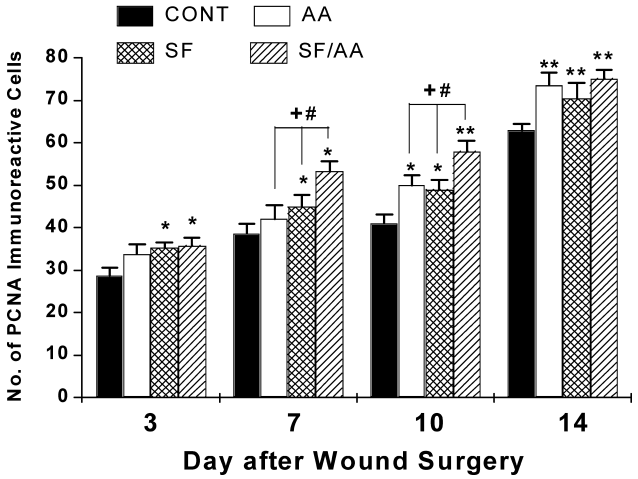

Fig. 4 The number of proliferating cell nuclear antigen (PCNA) immunoreactive cell. The value of PCNA positive cells was evaluated in silk fibroin (SF), alginate (AA) and silk fibroin-alginate (SF/AA) sponges-treated groups as well as Nu Gauze^{TM}-treated group (CONT). Each bar represented mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with CONT, $^{+}p < 0.05$ compared with AA and $^{\#}p < 0.05$ compared with SF.

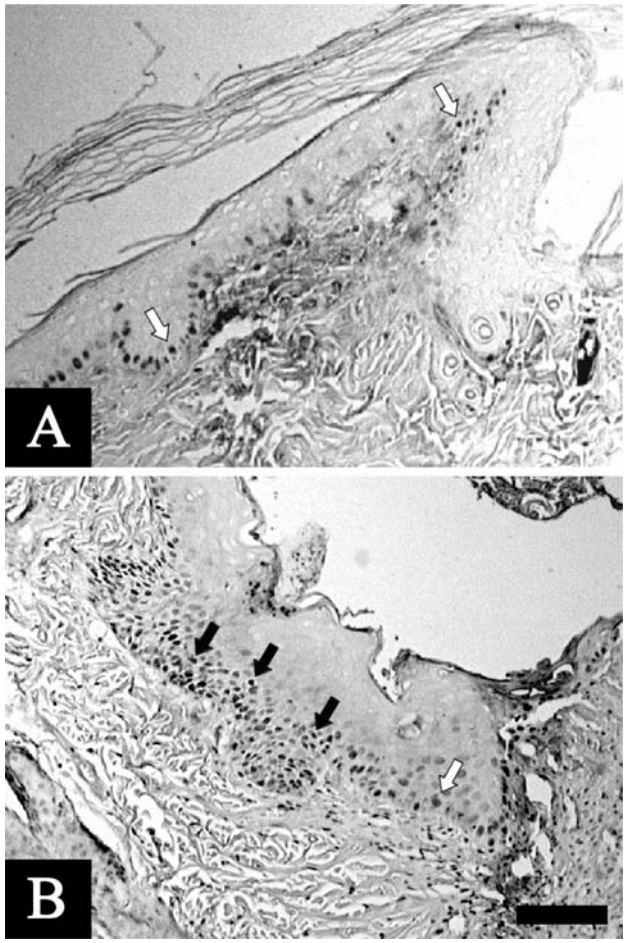

Fig. 5 The immunohistological observation of proliferating cell nuclear antigen (PCNA) expression in full-thickness excision wound at 7 days after treatment. (A) Nu Gauze-treated group (CONT). (B) silk fibroin-alginate (SF/AA)-treated group. White arrows; PCNA-positive cells expressed in single layer of epidermis, Black arrows; PCNA-positive cells in multi layer of epidermis. Original magnification ×200. Scale bar = 100 μm.

4. Discussion

In the present study, we showed that not only SF/AA-blended sponge but also separate SF and AA sponge treatment increased a wound healing rate as compared with that of CONT treatment. Recently, a number of studies have reported that SF might be a good candidate for the biomedical applications such as wound healing and scaffolds for tissue engineering [3, 4, 10, 13–15]. Min *et al.* reported that SF nanofibers had a notable effect on cell attachment and spreading of normal human keratinocytes and fibroblasts *in vitro* [13]. Moreover, SF from Bombyx mori domestic silkworm exhibited as high the attachment and growth of fibroblast cells as collagen did. SF from Antheraea pernyi wild silkworm also displayed much higher cell attachment and growth as compared with collagen [10]. These findings indicated that SF could promote collagen synthesis via activation of fibroblast. In addition, Santin *et al*. reported that the SF-induced activation of the mononuclear cells, measured as interleukin-1βproduction, was lower than reference materials such as poly(styrene) and poly(2-hydroxyethyl methacrylate) [14]. It was also reported that SF film treatment produced less inflammation and neutrophil-lymphocyte infiltration of the wounds than clinically used-Duo Active dressing [3]. Furthermore, it was investigated that lactose-SF conjugates could be applied as a scaffold for hepatocyte attachment [15]. These studies demonstrated that SF produced minimal adverse effects on immune system and its histocompatibility was excellent. In this regard, SF could give advantages as blending material that was useful for wound treatment. Recently, Yeo *et al*. also reported that PVA/Chitosan/Fibroin (PCF)-blended spongy sheets had a salient effect on wound healing of rats [4].

It has been studied that the application of AA as the wound dressing material could protect unhealed skin defect area from external stimuli and help to maintain the proper healing environment, and then could promote wound healing process [11, 16–20]. For example, when AA comes into contact with wound exudates, ion exchange occurs between the calcium ions of AA and the sodium ions in the exudates resulting in the formation of a gel on the surface of the wound bed. This hydrogel maintains an appropriate moist environment, which is considered to accelerate optimal wound healing [11, 18, 20]. Especially, calcium ion promotes homeostasis in the acute phase and influences the intracellular environment like cell migration at later stages of wound repair [16]. Moreover, calcium alginate increased the proliferation of fibroblasts but

delayed the proliferation of keratinocytes without influencing their motility in an in vitro wound construct [17]. These findings suggested that the calcium released by AA dressing might play critical roles in improvement of wound healing. The results of this study also indicated that single-handedly AA sponge produced significant wound healing effects.

Consequently, we designed this experiment to evaluate whether the SF/AA-blended sponge showed a synergic effect on wound healing processing. And it was revealed that SF/AA-blended sponge treatment produced the most prominent wound healing effect as compared with either SF or AA sponge treatment. However, it was uncertain which processes or mechanisms of wound healing were involved in the synergic effect of SF/AA-blended sponge. Accordingly, we performed histological staining for re-epithelialization and collagen deposition on wound healing state. Although SF/AA sponge treatments had significant effects on collagen deposition from 7 days after treatment as comparison to CONT treatment, its effect was not statistically different as compared with either that of SF or AA treatment group. However, SF/AA sponge treatment showed a significant synergic effect on re-epithelialization as compared with either that of SF or AA treatment group. These findings indicated that the synergic effect of SF/AA-blended sponge is mainly involved in the promotion of re-epithelialization rather than collagen deposition.

Furthermore, the synergic effect of SF/AA-blended sponge treatment on the re-epithelialization was confirmed utilizing immunohistochemistry for PCNA expression in the basal cell of hypertrophic epithelium. The number of PCNA positive cell in SF/AA sponge treated group was significantly increased in 7 and 10 days after sponge application as compared with that of either SF or AA group. This result suggested that the synergic healing effect of SF/AA sponge was mediated by the acceleration of re-epithelialization process via rapid proliferation of epithelial cell. It was demonstrated that the proliferation of epithelial cell could promote re-epithelialization, which accelerated wound repair process [16]. However, further study need to evaluate the precise mechanism of rapid epithelial cell proliferation induced by SF/AA sponge treatment.

5. Conclusions

The present study demonstrates that the wound healing effect of SF/AA-blended sponge is the best among other treatments including SF and AA treatments during the whole wound healing period. The healing effect of SF/AA-blended sponge is mediated by both re-epithelialization via rapid proliferation of epithelial cell and collagen deposition via fibroblasts proliferation. Especially, the synergic wound healing effect of SF/AA is involved in the acceleration of re-epithelialization. Based on these findings, SF/AA-blended sponge dressing may be clinically useful for skin wound treatment.

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