Evaluation of the wound healing process in rat skin using a hexanoyl group-modified alkaline-treated gelatin porous film

Running title: Wound healing process dressed with HxAlGltn

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ABSTRACT

The present study investigated the wound healing process in a rat skin wound model dressed with a hexanoyl group-modified alkaline-treated gelatin porous (HxAlGltn) film with angiogenesis-inducing and strong bioadhesive properties or a collagen sponge (CS). A circular 10-mm full-thickness skin defect was created on the backs of 8-week-old rats and then dressed with a HxAlGltn film (HxAlGltn group), collagen sponge (CS group), and no dressing (control group). Wound distances and areas were assessed macroscopically at specific time points after surgery, the lengths and thicknesses of the neo-epithelium histologically, and α-smooth muscle actin (α-SMA), collagen type I, and fibronectin immunohistochemically.

Wound distances and areas were significantly smaller in the HxAlGltn group than in the CS and control groups. The expression of α-SMA in myofibroblasts was significantly weaker in the HxAlGltn group than in the other groups on day 7. Furthermore,

angiogenesis was prominent on days 4 and 7 in the HxAlGltn group. No significant differences were observed in the length of the neo-epithelium between all groups, whereas the thickness of the neo-epithelium was greater in the HxAlGltn group than in the CS group.

The present results suggest the potential of the HxAlGltn film as a dressing material in the wound healing process of full-thickness skin defects because it reduces scar contraction and promotes angiogenesis more than CS.

Keywords: Hexanoyl group-modified alkaline-treated gelatin porous film; Collagen sponge; Scar contracture; Wound healing; Dressing material

INTRODUCTION

Oral and maxillofacial surgeries often cause tissue defects, including defects in the epithelium, and may result in scar contracture. Scar contracture in the wound healing process after oral and maxillofacial surgeries may cause facial anomalies, speech issues, and maxillary developmental disorders.[1] Thus, postoperative scar contracture is an important factor that may affect the quality of life of patients undergoing oral and maxillofacial surgeries.[2, 3]

The wound healing process has four stages: hemostasis, inflammation, proliferation, and maturation.[4, 5] Scar contracture is associated with the inflammatory and proliferative phases.[3] The inflammatory stage begins approximately 6 hours after injury. In this stage, various inflammatory cells secrete growth factors and inflammatory cytokines, which promote the growth of fibroblasts and vascular endothelial cells.[6] Wound contraction occurs in the latter half of the proliferative stage, in which granulation tissue starts to form.[4] Wound contraction is regarded as a defense response to the lost protective barrier of skin[7]; however, excessive wound contraction is one of the causes of postoperative scar contracture.[2, 3] Postoperative scar contracture has been

attributed to fibroblasts being induced to differentiate into myofibroblasts by inflammatory cytokines.[7] In the early wound healing process, myofibroblasts expressing α-smooth muscle actin (α-SMA) have been suggested to cause contraction and postoperative scar contracture.[3]

Various wound dressings that focus on the early closure of wounds have been produced.[8-10] In order to prevent postoperative scar contracture, a number of absorbable materials are used in surgeries to cover the epithelial defects of wounds.[11, 12] Wound dressings made of atelocollagen sponges are often used in oral and maxillofacial surgeries.[13, 14] Atelocollagen not only functions as a scaffold for the surrounding cells, but also promotes cell infiltration.

We previously examined the effects of different wound dressings on the early stages of the wound healing process, and reported that CS wound dressings inhibited wound contraction in the early wound area by suppressing the expression of α-SMA.[7] However, the inhibition of wound contraction by CS wound dressings was mild and epithelialization of the wound area was not promoted. On the other hand, the contribution of vascular endothelial cells to myofibroblast differentiation in the wound

area has been demonstrated.[15] Angiogenesis in the wound healing area is also considered to control the progression of wound healing. Thus, new dressing materials that suppress wound contraction, promote the neo-epithelium, and increase angiogenesis in the wound area are needed.

We developed a hexanoyl group-modified alkaline-treated gelatin film (HxAlGltn) with a porous structure using the salt-leaching method. A previous study reported that HxAlGltn showed strong adhesiveness to and induced angiogenesis in porcine intestinal surfaces and was biocompatible.[16] In an examination of transplantation into rat subcutaneous tissues, fast tissue infiltration and short degradation times were observed in HxAlGltn with a porous structure. The expression of the angiogenic marker CD34 was also increased around HxAlGltn.[17] We previously reported the advantages of HxAlGltn for angiogenesis *in vivo*. However, the influence of HxAlGltn on the wound healing process currently remains unclear.

In the present study, we used a rat surgical wound model and compared the wound healing process in full-thickness skin wounds dressed with HxAlGltn and CS.

MATERIALS AND METHODS

Animals

We used 8-week-old female Wistar rats purchased from Sankyo Laboratory Services Corporation (Tokyo, Japan) as the wound healing model in the present study. Rats were acclimated to our experimental environment for one week before experimentation and housed in individual cages. Rats were maintained and treated according to the protocols of the Division of Laboratory Animal Science at the Natural Science Center for Research and Education at Kagoshima University. The present study was approved by the Division of Laboratory Animal Science at the Natural Science Center for Research and Education at Kagoshima University (No. D16001), and performed according to the Japanese Government Animal Protection and Management Law.

Wounding, treatment, and experiments

Surgical wounds were performed as previously reported by Kibe et al.[7, 18] Anesthesia was induced using pentobarbital (10 mg/kg) and maintained with isoflurane

(1.5%), and the hair on the back was shaved. Circular 10-mm full-thickness skin wounds were created in the center of the back using a biopsy punch and given markings around the wound. In the control group (N=3), wounded areas were not covered with any dressing. In the HxAlGltn (N=3) and CS (N=3) groups, the wounded areas were covered with HxAlGltn and CS, respectively, and fixed using a rat jacket. We macroscopically monitored wound healing by taking digital photographs at the indicated time points (days 0, 4, 7, 14, 21, and 28 after surgery). The wound area in the digital photograph was calculated using the image analysis software ImageJ. Mice were injected intraperitoneally with an overdose of nembutal for sacrifice, and the wounded skin area was collected post-mortem for further experiments.

Measurement of wound contraction

Wound contraction was assessed as described in a previous study.[7] In brief, wound contraction was evaluated by measuring the X- and Y-wound distances between markings before surgery and on days 0, 4, 7, 14, 21, and 28 after surgery. Wound photographs were taken with a millimeter ruler for calibration and wound images were

then exported to ImageJ. Wound areas were manually tracked and calculated by a researcher blinded to group affiliations. Average measurements of wound areas were used in the statistical analysis.

Histological and immunohistochemical observations

Histological and immunohistochemical observations were performed as described previously.[7, 19, 20] In brief, after fixing excised skin sections in 10% formalin for 48 hours, fixed tissues were dehydrated and embedded in paraffin. Fixed tissues were sectioned into 4-μm-thick slices, deparaffinized in xylene, and rehydrated. The morphology of wound tissue was observed in hematoxylin and eosin-stained sections. The distance and thickness of neo-epithelial tissue were measured in these sections using ImageJ.

α-SMA, fibronectin, and collagen type I were immunohistochemically examined. An anti-α-SMA antibody (a mouse monoclonal antibody) was purchased from Calbiochem (Darmstadt, Germany), and anti-fibronectin (a mouse monoclonal antibody) and anti-collagen type I (a rabbit polyclonal antibody) antibodies from Abcam

(Tokyo, Japan). In brief, sections were incubated with a primary antibody conjugated to fluorescein (1:250 dilution; F3777; Sigma-Aldrich, Japan). Sections were then incubated with a second antibody, a rabbit anti-fluorescein antibody (1:750 dilution; A889; Molecular Probes, Eugene, OR, USA), and then with biotinylated goat-anti-rabbit IgG (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). Sections were subsequently incubated with a diluted ABC-alkaline phosphatase complex (Vector Laboratories, CA, USA) and color was developed in the presence of Vector black alkaline phosphatase substrate (Vector Laboratories). Control sections were treated by the same methods, except for the primary antibody. Immunostaining was evaluated by one of the authors (T. K.). Areas that positively stained for α-SMA, fibronectin, and collagen type I were then evaluated by software (the Hybrid cell count system, Keyence, Tokyo, Japan), which showed the ratio of positive cell numbers among all cells in the region being observed.

Statistical analysis

Statistical analyses were performed on evaluations of the wound area, X- and Y-

wound distances, neo-epithelium lengths and thicknesses, and the expression of α-SMA, collagen type I, and fibronectin. The Kruskal-Wallis test and Steel-Dwass test were used to assess the significance of differences. P-values less than 0.05 were considered to be significant. Statistical analyses were performed using GraphPad Prism 6 (San Diego, CA).

RESULTS

Dressing materials and markings

HxAlGltn was prepared by the salt-leaching method as reported previously.[17] Briefly, HxAlGltn was dissolved in 10% L-lactic acid (LA; Wako Pure Chemical Industries, Ltd., Tokyo, Japan)-dimethyl sulfoxide (DMSO) solvent to prepare 25 w/v% HxAlGltn solution. Trisuccinimidyl citrate (TSC) was subsequently added to crosslink HxAlGltn molecules. Sodium chloride (NaCl: Wako Pure Chemical Industries, Ltd., Tokyo, Japan) [for liquid-solid ratios of 4/1 (w/w)] was added to the solution and mixed to uniformly disperse NaCl. The resulting solutions were packaged in bags with zippers (Unipack, Seisan Nipponsha Ltd., Tokyo, Japan). After overnight crosslinking, the

NaCl-HxAlGltn gels obtained were soaked in 4 °C ultra-pure water for 3 days to remove unreacted TSC, NaCl, and LA-DMSO. Thereafter, HxAlGltn was obtained by lyophilization of the resulting porous hydrogels for 3 days.

The other dressing material used was CS (TERUDERMIS, Olympus Terumo Biomaterials Corp., Japan), comprising collagen derived from the dermis of young cows (Fig. 1A).[13] It consisted of lower and upper layers; the lower layer was CS, while the upper layer was a silicon membrane. In the present study, we only used the lower layer to compare collagen with HxAlGltn.

As shown in Figure 1B, circular 10-mm full-thickness skin wounds were created in the center of the back. Figure 1C shows the margins of the wound area. X indicates the distance between markings perpendicular to the body axis of the rat, and Y the distance between markings parallel to the body axis of the rat.

Time-dependent changes in wound distances and wound areas

Changes in the macroscopic appearance of the wound area between days 0, 4, 7, 14, 21, and 28 are shown in Figure 2A. Wound distances between the four reference

punctures around the wound area were measured (X- and Y-lengths) and wound areas on each day are shown in Figure 2B and Figure 2C, respectively.

Regarding the early wound healing process, the X-length was reduced by approximately 68-75% in the control and CS groups and by 86% in the HxAlGltn group on day 7; the X-length was significantly greater in the control and CS groups than in the HxAlGltn group ($p<0.05$). The Y-length was reduced by 75% in the control group, by 81% in the HxAlGltn group, and by 88% in the CS group on day 7; the Y-length was significantly greater in the control group than in the HxAlGltn and CS groups ($p<0.05$).

Regarding the late wound healing process on day 28, the X-length was reduced by approximately 55% in the control and CS groups, and by 70% in the HxAlGltn group; the X-length was significantly greater in the control and CS groups than in the HxAlGltn group ($p<0.05$). The Y-length was reduced by 55% in the control group, by 70% in the HxAlGltn group, and by 63% in the CS group; the Y-length was significantly greater in the control group than in the HxAlGltn group ($p<0.05$).

The wound area was significantly smaller in the control group than in the HxAlGltn and CS groups on day 7 ($P<0.05$, Fig. 2C). All wounds were approximately

closed on day 21.

Time-dependent changes in the length and thickness of epithelial neogenesis

The growth position of new epithelial cells in the wound is shown in Figure 3A. The neo-epithelium was observed on the surface of the wound in the control group and on the dressing material covering the wound surface in the HxAlGltn and CS groups. The length of the neo-epithelium was approximately 1000 μm in all groups on day 7; no significant differences were observed between the three groups (Fig. 3B). The thickness of the neo-epithelium on day 28 was 50-60 μm in the control and HxAlGltn groups and 20 μm in the CS group. The neo-epithelium was significantly thicker in the control and HxAlGltn groups than in the CS group ($p<0.05$, Fig. 3B).

Evaluation of myofibroblasts and angiogenesis

The expression of α-SMA in the wound on day 7 is shown in Figure 4A. α-SMA-positive cells were observed in both vessels and myofibroblasts in the wound area (Fig. 4A). α-SMA-positive cells were separately evaluated as angiogenesis with ductal

structures and myofibroblasts without these structures. The ratio of positive staining for angiogenesis among the whole area observed was significantly greater in the HxAlGltn group than in the other groups ($p<0.05$, Fig. 4B). On the other hand, the ratio of positive staining areas for myofibroblasts among the whole observed area was significantly greater in the control group than in the other groups on day 7 ($p<0.01$, Fig. 4C). Furthermore, the ratio of positive staining areas for myofibroblasts was significantly smaller in the HxAlGltn group than in the CS group ($p<0.05$, Fig. 4C).

Evaluations of angiogenesis by counting the number of new vessels, which were stained by α-SMA, in the wound area revealed that the number of vessels was the highest in the HxAlGltn group on days 4 and 7 (Fig. 4D). On day 28, the number of blood vessels in the HxAlGltn group was the smallest; however, large new blood vessels were observed.

Evaluation of the expression of fibronectin and collagen type I

Figure 5A shows the expression of fibronectin on day 7 in the wound area. Fibronectin-positive cells were observed in granulation tissue in the wound area in all

groups (Fig. 5A). The ratio of positive staining areas for fibronectin in the wound area was significantly greater in the control group than in the other groups on days 4 and 7 ($p<0.05$). On day 28, this ratio was greater in the HxAlGltn group than in the other groups ($p<0.05$, Fig. 5B).

Figure 5C shows the expression of collagen type I on day 7 in the wound area. The ratio of positive staining areas for collagen type I in the wound area was greater in the HxAlGltn group followed by the CS group; a significant difference was observed between the HxAlGltn and control groups ($p<0.01$) and between the HxAlGltn and CS groups throughout the study period ($p<0.05$) (Fig. 5D). The number of neutrophils, macrophages, and lymphocytes, which is an index of the inflammatory response, did not significantly differ among the three groups (Fig. 5E).

DISCUSSION

We previously investigated the characteristics of various artificial dressing materials for covering wound defects in order to facilitate better wound healing in the oral

and maxillofacial surgery field.[7] We demonstrated that CS wound dressings inhibited wound contraction by suppressing the expression of α-SMA in the early stage of the wound healing process. However, in CS wound dressings, the inhibition of wound contraction was mild and epithelialization of the wound area was not promoted.[7] Since angiogenesis in the wound healing area is considered to control the progression of wound healing, new dressing materials that suppress wound contraction, promote the neo-epithelium, and increase angiogenesis in the wound area are needed. Therefore, in the present study, we focused on a novel dressing material, HxAlGltn, with angiogenesis-inducing and strong bioadhesive properties.[17, 21, 22] The results obtained on the effects of HxAlGltn on wound healing are presented below.

Effects of HxAlGltn on contraction in full-thickness skin wounds

The suppression of wound contraction during the wound healing process was significantly greater in the HxAlGltn group than in the control and CS groups.

We previously compared the early healing process between absorbable polyglycolic acid (PGA) sheets and CS, and found that PGA suppressed wound contraction.[7] Since PGA

sheets are slowly degraded *in vivo*, removal of the PGA sheet resulted in the peeling off of the neo-epithelium from the wound area, which was demonstrated in long-term observations of the wound healing process. Therefore, we compared HxAlGltn and CS in the present study. HxAlGltn more strongly suppressed the expression of α-SMA in myofibroblasts than the control and CS. This result suggested that the suppression of wound contraction with HxAlGltn was due to a decrease in α-SMA-positive myofibroblasts, which promote wound contraction. Further studies involving cell biological analyses are needed to elucidate the mechanisms underlying reductions in myofibroblast numbers.

Effects of HxAlGltn on the neo-epithelium

HxAlGltn was confirmed as a foothold for epithelial regeneration, as demonstrated by epithelial neogenesis on the surface of HxAlGltn. The renewed epithelium of the wound area in the HxAlGltn group was observed on the surface of HxAlGltn. Similar results were obtained in the control and CS groups. Furthermore, a thicker epithelium formed with HxAlGltn than with CS, and the thickness of the renewed epithelium was

equivalent to that of the healthy epithelium. These results suggest that HxAlGltn functions as a healthy scaffold for epithelial cells.

In contrast, the length of epithelial neogenesis with HxAlGltn was not significantly different from those with the control and CS. The closure of a wound in the living body, which represents healing, generally involves two elements: wound contraction and epithelial neogenesis.[7] The present results suggest that the delay observed in the reduction of the wound area in the HxAlGltn group was not the effect of epithelial neogenesis, but was caused by the inhibited contraction of the wound area. In the present study, the thickness of the healthy epithelium was approximately 60 μm. Therefore, the thickness of the renewed epithelium in the HxAlGltn group may be regarded as epithelialization of a more physiological thickness than that in the CS group.

Fibronectin is an adhesion molecule that is important in extracellular matrix (ECM) formation and re-epithelialization in the wound healing process.[23] Among the three groups examined in the present study, fibronectin was the most strongly expressed in the control group. In the HxAlGltn group, fibronectin was not expressed in the early wound healing stage. This may account for the lack of an increase in the length of

epithelial neogenesis.

Effects of HxAlGltn on angiogenesis and collagen production

The third observed characteristic of HxAlGltn was that blood vessel-rich granulation tissue formed in the wound area, and the expression of collagen type I was increased. Angiogenesis is considered to be important for proper healing, and is necessary for the transport of nutrition, immune cells, and oxygen to the wound area.[4] The majority of newly generated capillaries are subsequently degraded and capillary density becomes similar to that of normal skin.[15] In the present study, angiogenesis was significantly weaker on day 28 in the HxAlGltn group than in the two other groups. These results suggest that wound healing progressed at a faster rate in the HxAlGltn group than in the other two groups. We previously reported that HxAlGltn films bound endogenous vascular endothelial growth factor (VEGF) and promoted vascular induction. [16, 17] The enhancement in angiogenesis in the present study was attributed to the HxAlGltn film binding endogenous VEGF.

Moreover, the proliferation of fibroblasts and production of collagen in the wound

area are important for restoring defects in mesenchymal tissue. Previous studies reported that wound dressing may promote fibroblast migration and proliferation.[24, 25] Thus, in an evaluation of collagen type I, we found that its expression was significantly stronger in the HxAlGltn group in all observation periods than in the other groups. This result suggests that wound healing in the HxAlGltn group progressed quickly and that HxAlGltn has the ability to promote the maturation phase of wound healing by enhancing the production of collagen in defective mesenchymal tissue.

Although HxAlGltn did not promote epithelial regeneration, a physiologically thick renewed epithelium, angiogenesis, and the production of collagen type I were observed. These results suggest that HxAlGltn is a useful wound dressing that functions as a healthy scaffold for epithelial cells. However, since the promotion of epithelial regeneration is important for wound healing, further modifications, such as the addition of epithelial growth factor, are needed.

Limitations

The present study had some limitations. We were unable to completely control the

movement of rats, which may have affected the wound healing process. Furthermore, the present study had an observation period of up to 28 days. A longer time period may be needed to observe scar contracture.

CONCLUSION

The present results suggest that HxAlGltn films are more useful than CS as a dressing material to reduce scar contraction and promote angiogenesis in the wound healing process of full-thickness skin defects.

REFERENCES

1. Naqvi ZA, Shivalinga BM, Ravi S, Munawwar SS. Effect of cleft lip palate repair on craniofacial growth. J Orthod Sci 2015; 4:59-64.

2. Yura S, Ooi K, Izumiyama Y. Repair of oral mucosal defects using artificial dermis: factors related to postoperative scar contracture. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011; 112:161-163.

3. Hinz B. Myofibroblasts. Exp Eye Res 2016; 142:56-70.

4. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. Nature 2008; 453:314-321.

5. Deyhimi P, Khademi H, Birang R, Akhoondzadeh M. Histological evaluation of wound healing process after photodynamic therapy of rat oral mucosal ulcer. J Dent (Shiraz) 2016; 17:43-48.

6. Michalczyk ER, Chen L, Fine D, Zhao Y, Mascarinas E, Grippo PJ, DiPietro LA. Pigment epithelium-derived factor (PEDF) as a regulator of wound angiogenesis. Sci Rep 2018; 8:11142.

7. Kibe T, Koga T, Nishihara K, Fuchigami T, Yoshimura T, Taguchi T, Nakamura

N. Examination of the early wound healing process under different wound dressing conditions. Oral Surg Oral Med Oral Pathol Oral Radiol 2017; 123:310-319.

8. Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med 1999; 341:738-746.

9. Li J, Chen J, Kirsner R. Pathophysiology of acute wound healing. Clin Dermatol 2007; 25:9-18.

10. Khan I, Arany P. Biophysical approaches for oral wound healing: emphasis on photobiomodulation. Adv Wound Care (New Rochelle) 2015; 4:724-737.

11. Omura S, Mizuki N, Horimoto S, Kawabe R, Fujita K. A newly developed collagen/silicone bilayer membrane as a mucosal substitute: a preliminary report. Br J Oral Maxillofac Surg 1997; 35:85-91.

12. Okuyama K, Yanamoto S, Naruse T, Sakamoto Y, Rokutanda S, Ohba S, et al. Clinical complications in the application of polyglycolic acid sheets with fibrin glue after resection of mucosal lesions in oral cavity. Oral Surg Oral Med Oral Pathol Oral Radiol 2018; 125:541-546.

13. Matsumoto Y, Ikeda K, Yamaya Y, Yamashita K, Saito T, Hoshino Y, Koga T, Enari H, Suto S, Yotsuyanagi T. The usefulness of the collagen and elastin sponge derived from salmon as an artificial dermis and scaffold for tissue engineering. Biomed Res 2011; 32:29-36.

14. Kuwata T, Shinohara S, Takenaka M, Oka S, Chikaishi Y, Hirai A, Kuroda K, So T, Tanaka F. The impact of covering the bulla with an absorbable polyglycolic acid (PGA) sheet during pneumothorax surgery. Gen Thorac Cardiovasc Surg 2016; 64:558-560.

15. Miyazaki T, Haraguchi S, Kim-Kaneyama JR, Miyazaki A. Endothelial calpain systems orchestrate myofibroblast differentiation during wound healing. FASEB J 2019; 33:2037-2046.

16. Yoshizawa K, Taguchi T. Enhanced bonding strength of hydrophobically modified gelatin films on wet blood vessels. Int J Mol Sci 2014; 15:2142-2156.

17. Yoshizawa K, Mizuta R, Taguchi T. Enhanced angiogenesis of growth factor-free porous biodegradable adhesive made with hexanoyl group-modified gelatin. Biomaterials 2015; 63:14-23.

18. Mirastschijski U, Haaksma CJ, Tomasek JJ, Agren MS. Matrix metalloproteinase inhibitor GM 6001 attenuates keratinocyte migration, contraction and myofibroblast formation in skin wounds. Exp Cell Res 2004; 299:465-475.

19. Kibe T, Fuchigami T, Kishida M, Iijima M, Ishihata K, Hijioka H, Miyawaki A, Semba I, Nakamura N, Kiyono T, Kishida S. A novel ameloblastoma cell line (AM-3) secretes MMP-9 in response to Wnt-3a and induces osteoclastogenesis. Oral Surg Oral Med Oral Pathol Oral Radiol 2013; 115:780-788.

20. Mirastschijski U, Schnabel R, Claes J, Schneider W, Agren MS, Haaksma C, Tomasek JJ. Matrix metalloproteinase inhibition delays wound healing and blocks the latent transforming growth factor-beta1-promoted myofibroblast formation and function. Wound Repair Regen 2010; 18:223-234.

21. Takayama T, Taguchi T, Koyama H, Sakari M, Kamimura W, Takato T, Miyata T, Nagawa H. The growth of a vascular network inside a collagen-citric acid derivative hydrogel in rats. Biomaterials 2009; 30:3580-3587.

22. Matsuda M, Ueno M, Endo Y, Inoue M, Sasaki M, Taguchi T. Enhanced tissue penetration-induced high bonding strength of a novel tissue adhesive composed

of cholesteryl group-modified gelatin and disuccinimidyl tartarate. Colloids Surf B Biointerfaces 2012; 91:48-56.

23. Lenselink EA. Role of fibronectin in normal wound healing. Int Wound J 2015; 12:313-316.

24. Moura LI, Dias AM, Suesca E, Casadiegos S, Leal EC, Fontanilla MR, Carvalho L, de Sousa HC, Carvalho E. Neurotensin-loaded collagen dressings reduce inflammation and improve wound healing in diabetic mice. Biochim Biophys Acta 2014; 1842:32-43.

25. Zhao X, Wu H, Guo B, Dong R, Qiu Y, Ma PX. Antibacterial anti-oxidant electroactive injectable hydrogel as self-healing wound dressing with hemostasis and adhesiveness for cutaneous wound healing. Biomaterials 2017; 122:34-47.

FIGURE LEGENDS

Figure 1 Dressing materials and surgical wound creation in rats. (A) A hexanoyl group-modified alkaline-treated gelatin porous film (HxAlGltn) and collagen sponge (CS). (B) A 10-mm full-thickness skin defect was made in the central area of the back of each rat as shown and the margins of the wounded area were marked. (C) Measurements of the margins of the wound are shown. X indicates the distance between the markings perpendicular to the body axis of the rat, and Y indicates the distance between the markings parallel to the body axis of the rat.

Figure 2 Changes in lengths (X and Y) between wound margins. (A) Representative images showing the macroscopic appearance of the wound on days 0, 4, 7, 14, 21, and 28 in the control, HxAlGltn, and CS groups. (B) The X-length is the distance between wound markings perpendicular to the body axis of the rat. The Y-length is the distance between wound markings parallel to the horizontal body axis of the rat. The percentage of each measurement from the day 0 measurement is shown. (C) The area of the wound was separately calculated from digital images using the image analysis software, ImageJ.

Error bars indicate the standard error of the mean. *indicates $p<0.05$.

CS: Collagen sponge; HxAlGltn: hexanoyl group-modified alkaline-treated gelatin porous film

Figure 3 Epithelial neogenesis in the wound. (A) Representative images show the growth position of new epithelial cells in a wound on day 7 in the control, HxAlGltn, and CS groups. Hematoxylin and eosin staining was used, and the scale bar indicates 200 μm. Black arrows indicate new epithelial cells. White arrows indicate the initial wound edge. (B) The neo-epithelium length was measured as the distance between the initial wound edge and observed re-epithelialized leading edge in histological sections.

CS: Collagen sponge; HxAlGltn: hexanoyl group-modified alkaline-treated gelatin porous film

Figure 4 α-Smooth muscle actin (α-SMA) expression in the wound. (A) Representative images show α-SMA expression in the wound on day 7 in the control, HxAlGltn, and CS groups. The scale bar indicates 200 μm. (B) Comparison of the ratio of the positive

staining of angiogenesis among the whole observed area on days 4, 7, and 28. *indicates $p<0.05$. (C) Comparison of the ratio of the positive staining of myofibroblasts among the whole observed area on days 4, 7, and 28. *indicates $p<0.05$. **indicates $p<0.01$. (D) Comparison of the number of new vessels, which were stained by α-SMA, in the wound area on days 4, 7, and 28. *indicates $p<0.05$.

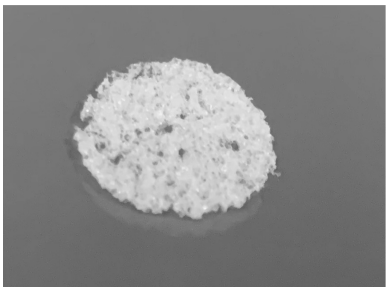
CS: Collagen sponge; HxAlGltn: hexanoyl group-modified alkaline-treated gelatin porous film

Figure 5 Fibronectin and collagen type I expression in the wound. (A) Representative images show fibronectin expression in the wound on day 7 in the control, HxAlGltn, and CS groups. The scale bar indicates 200 μm. (B) Comparison of the ratio of the positive staining of fibronectin among the whole observed area on days 4, 7, and 28. *indicates $p<0.05$. (C) Representative images show collagen type I expression in the wound area on day 7 post-wounding in the control, HxAlGltn, and CS groups. The scale bar indicates 200 μm. (D) Comparison of the ratio of the positive staining of collagen type I among the whole observed area on days 4, 7, and 28. *indicates $p<0.05$. **indicates $p<0.01$.

CS: Collagen sponge; HxAlGltn: hexanoyl group-modified alkaline-treated gelatin porous film (E) Comparison of the number of neutrophils, macrophages, and lymphocytes among the whole observed area on days 4, 7, and 28.

Fig. 1 Koga et al.

A



HxAlGltn

CS

B

C

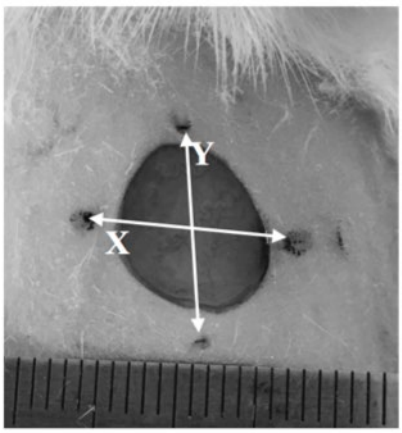
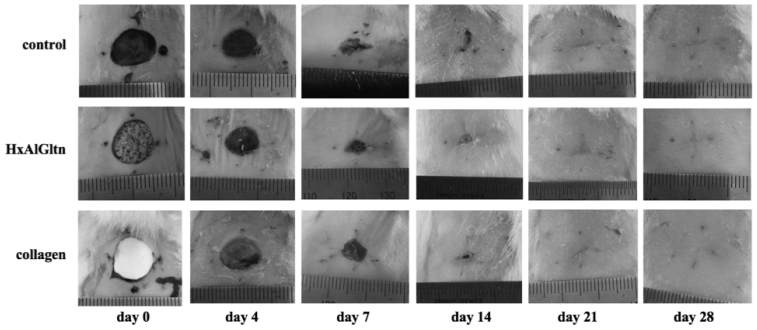


Fig. 2 Koga et al.

A



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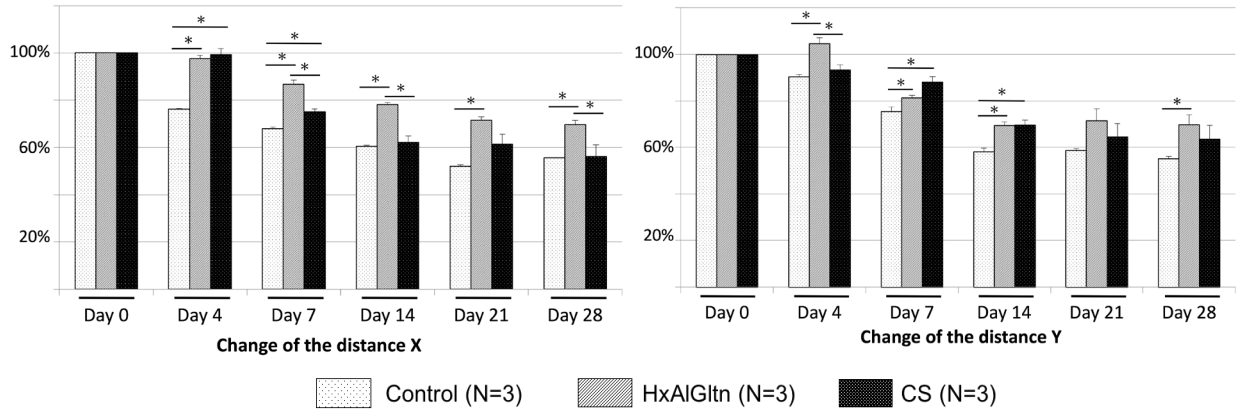

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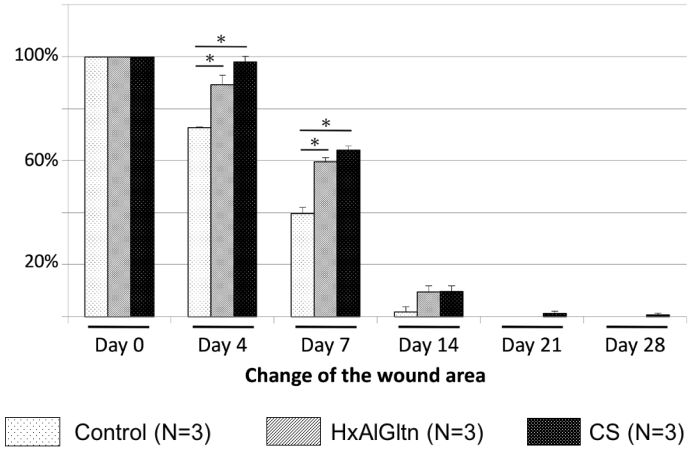
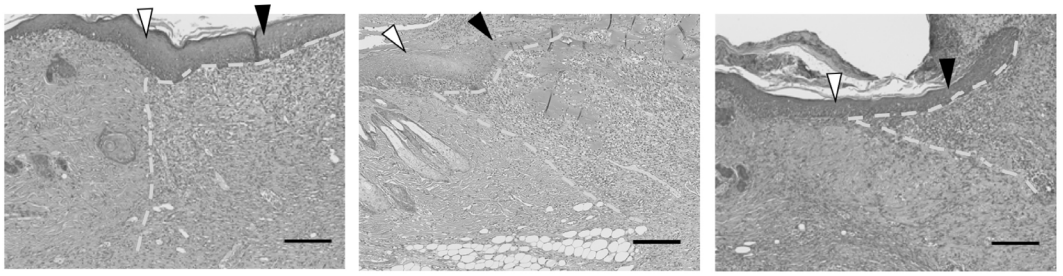


Fig. 3 Koga et al.

A



The growth position of new epithelial cells in a wound area

B

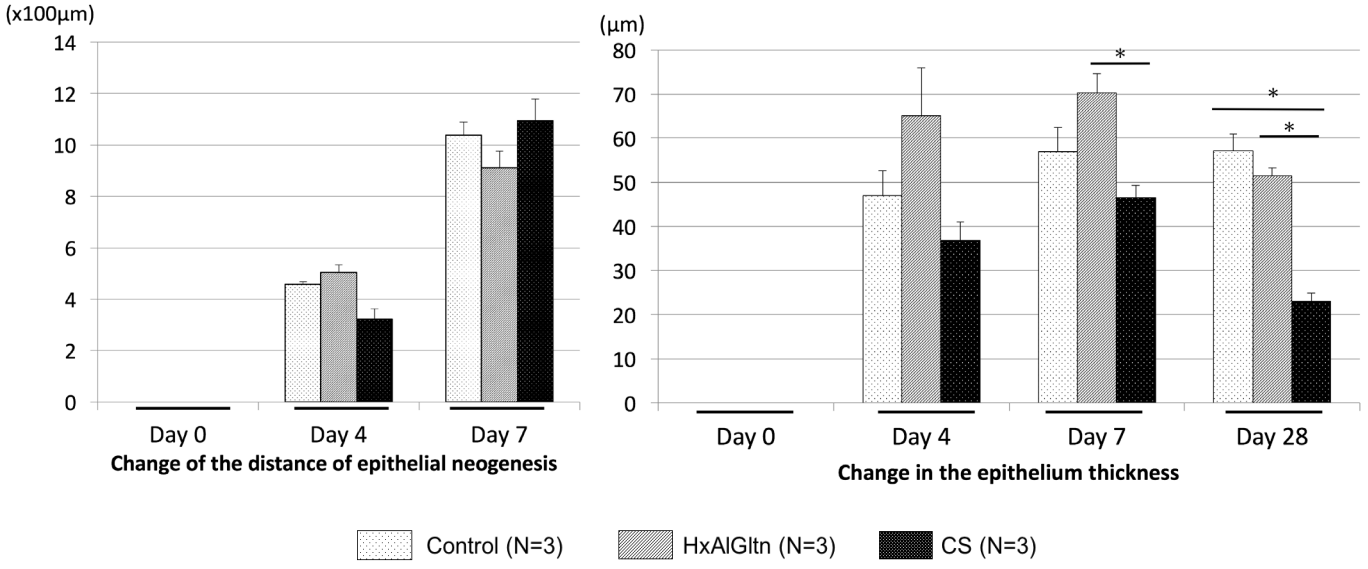
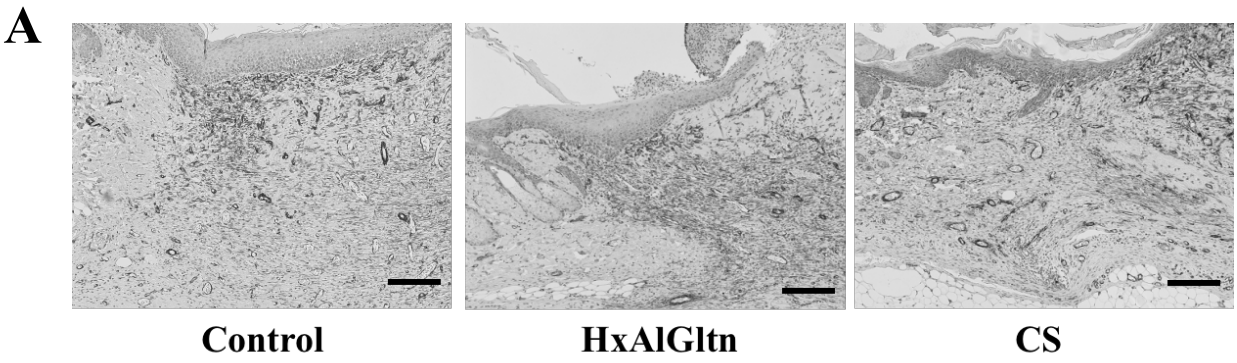
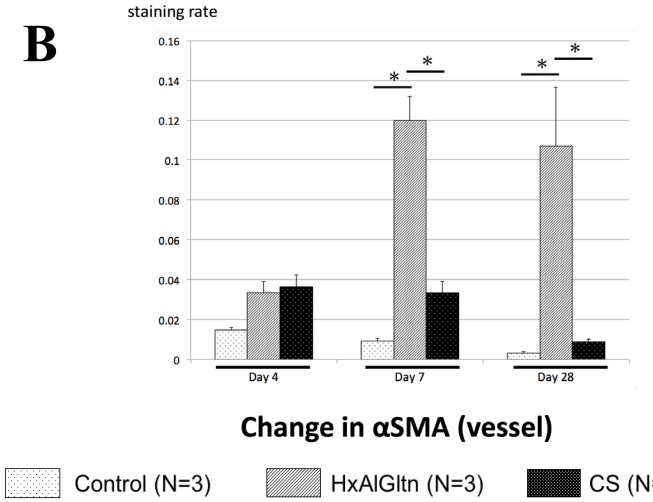
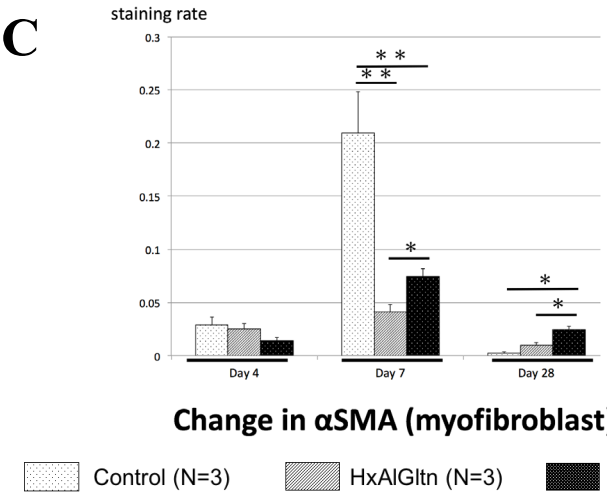


Fig. 4 Koga et al.







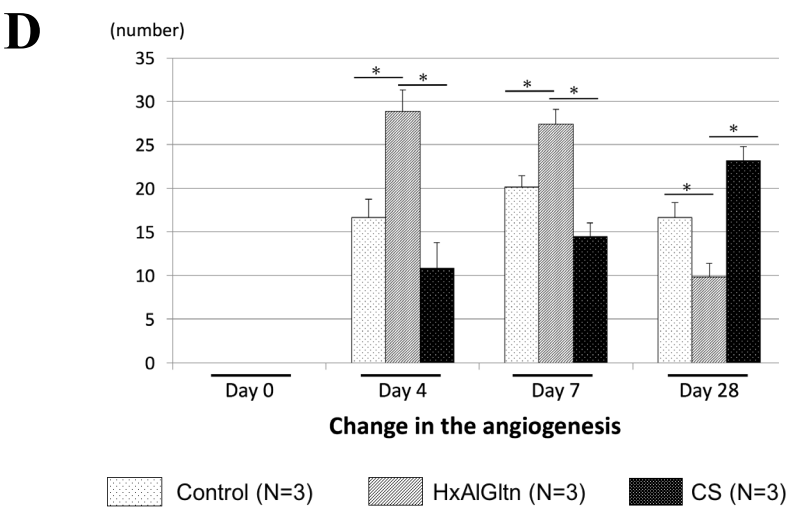
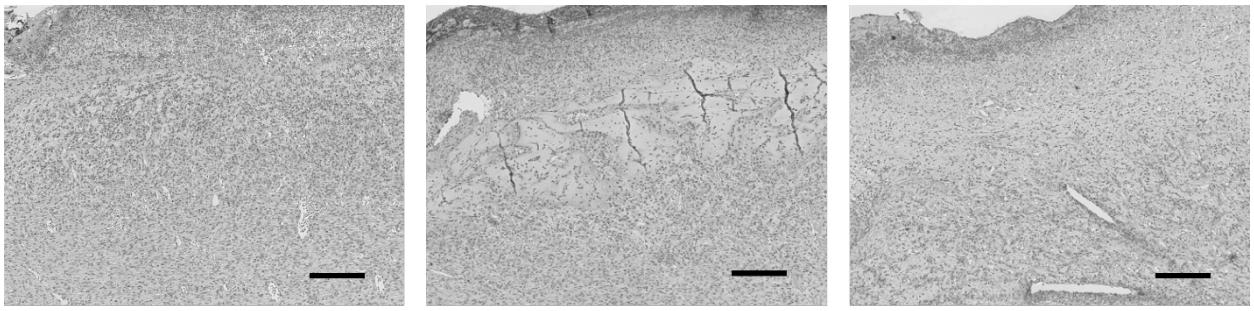


Fig. 5 Koga et al.

A



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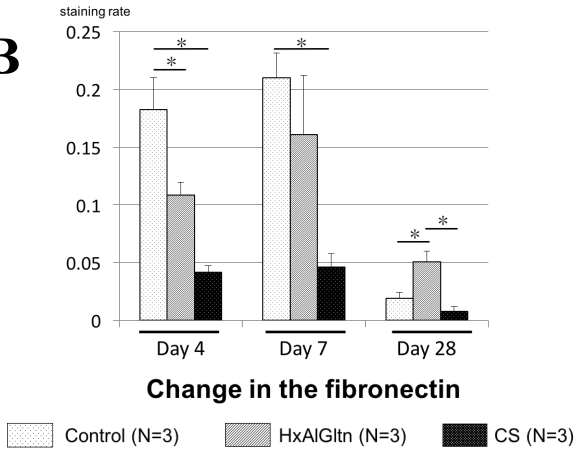

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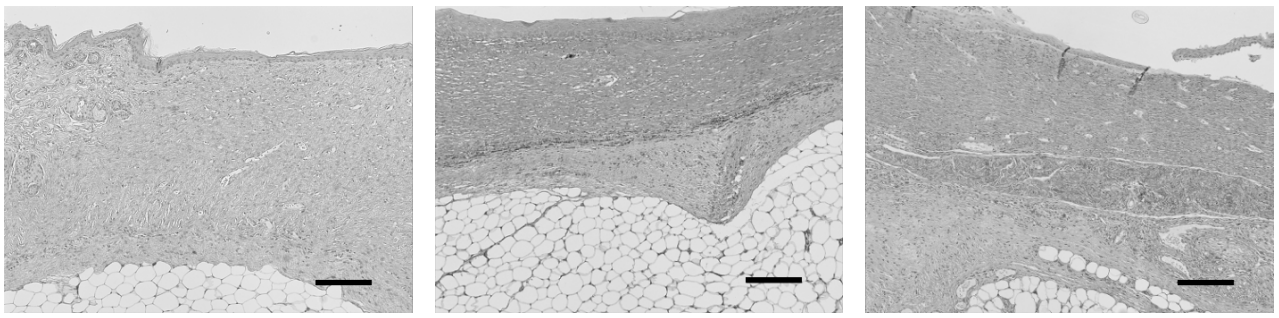

D