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Original Article

Efficacy of gelatin hydrogels incorporating triamcinolone acetonide for prevention of fibrosis in a mouse model

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ABSTRACT

Introduction: Triamcinolone acetonide (TA), a steroid, is often used clinically to prevent dysfunctions associated with fibrosis. The objective of this study was to examine whether TA can be suspended in a gelatin sheet for tissue engineering using a mouse skin wound model.
Methods: TA was suspended in biodegradable gelatin and freeze-dried in a sheet form. The sheet was analyzed for homogeneity and controlled release of TA by high-performance liquid chromatography. We made two skin wounds on the dorsal side of mice. Gelatin sheets with TA (TA sheet) and without TA (control sheet) were attached to each skin wound. To determine the efficacy of the prepared TA sheet on the skin wounds, TA-sheet versus TA-injection experiments were conducted. Hematoxylin and eosin staining was performed to assess the grade of epithelialization and alpha smooth muscle actin (α-SMA) immunohistochemical staining was conducted to evaluate myofibroblast infiltration.
Results: In the TA-release test *in vitro*, 7.7 ± 2.3% of TA was released from the sheet by 24 h. After replacing the initial phosphate-buffered saline (PBS) with collagenase PBS, the amount of released TA increased over time. The wound area/original skin wound area after 15 days with the TA sheet was significantly larger than that with the control sheet (26.9 ± 5.5% vs 10.7 ± 2.6%, $p = 0.023$). The α-SMA positive area/whole area with the TA sheet was significantly lower than that with the control sheet (4.65 ± 0.66% vs 7.24 ± 0.7%, $p = 0.023$). Furthermore, the α-SMA positive area/whole area with the TA sheet was significantly lower than that with TA injection (5.32 ± 0.45% vs 7.93 ± 0.75%, $p = 0.013$).
Conclusions: We developed a TA sheet and confirmed both the homogeneity of the suspended TA and controlled-release of the TA in the presence of collagenase *in vitro*. The TA sheet caused less myofibroblast infiltration into the tissue than the control sheet or TA injection did.

Abbreviations: TA, triamcinolone acetonide; α-SMA, alpha smooth muscle actin; PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor; TGF-β, transforming growth factor beta; IL-1, interleukin-1.

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1. Introduction

Fibrosis is a component of pathologic remodeling in many tissues and contributes to clinical disease. Fibrosis can negatively affect tissues, resulting in dysfunctions in various organs [1]. For example, skin contracture formation due to burn injury leads to a limited range of motion; esophageal stricture due to fibrosis after endoscopic mucosal resection for esophageal cancers leads to dysphagia [2]; and stricture formation from Crohn's disease, a refractory inflammatory bowel disease, causes gastrointestinal obstruction [3].

Steroid therapy is often used to treat or prevent severe fibrosis. Steroids inhibit fibrosis by preventing the migration and activation of inflammatory cells and myofibroblasts. Steroids modulate wound healing through their anti-inflammatory effects by decreasing prolyl hydroxylase activity and amplifying collagenase activity, thereby reducing tissue collagen content [4]. However, systemic steroid therapy can produce adverse effects, such as hyperglycemia, susceptibility to infection, and adrenal failure. Therefore, it is desirable to administer steroids as locally as possible.

Gelatin is a biodegradable material that has been used extensively for food, pharmaceutical, and medical purposes. The biological safety of gelatin has been demonstrated in numerous practical applications. It has been reported that using gelatin for controlled release can augment the therapeutic effects of drugs, such as pioglitazone for wound healing [5], simvastatin for bone regeneration [6], and neuropeptide substance P for angiogenesis [7], among other applications.

Based on the controlled-release properties of gelatin and effectiveness of steroids in preventing fibrosis, we predicted that the local administration of triamcinolone acetonide (TA)-infused gelatin could effectively prevent fibrosis in various organs. To the best of our knowledge, there have been no prior reports on the use of steroid-loaded gelatin sheets to prevent severe fibrosis. Thus, the objective of this study was to examine whether TA can be suspended in gelatin for tissue engineering.

2. Materials and methods

2.1. Preparation of TA gelatin sheets

Pig skin gelatin, with a molecular weight of 100,000 Da and an isoelectric point of 5.0, was kindly supplied by Nitta Gelatin Co. (Osaka, Japan).We used TA from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Gelatin (500 mg) was dissolved in 5 mL double-distilled water. Twenty milligrams of TA was suspended in 1 mL double-distilled water. These solutions were mixed well to homogeneity and rapidly frozen in liquid nitrogen followed by freeze-drying for 72 h to obtain the TA gelatin sheet (TA sheet). The sheet ($10 \times 10 \times 2$ mm^3) was crosslinked by dehydrothermal treatment at 140 °C for 48 h in a vacuum oven and sterilized with ethylene oxide. We also produced a gelatin sheet without TA as described above (control sheet).

2.2. High-performance liquid chromatography (HPLC)

To evaluate the homogeneity and quantity of TA suspended in the sheet, the sheet was cut into 4 pieces and each piece was placed in 2 mL methanol (Nacalai Tesque, Inc. Kyoto, Japan) for 72 h. The concentration of TA in the methanol was analyzed by HPLC (Prominence LC-20AT, Shimadzu, Kyoto, Japan), using a COSMOSIL(R) 5C18-AR-II Packed Column (4.6 mm I.D. × 250 mm, Nacalai Tesque, Inc.) and column temperature of 37 °C. The mobile phases were acetonitrile (Nacalai Tesque, Inc.) at 0.55 mL/min and 10 mM ammonium acetate (Wako Pure Chemical Industries, Ltd.) at 0.45 mL/min. The absorbance of the eluate was measured over 30 min at a wavelength of 260 nm and the TA concentration was determined by using a calibration curve that had been prepared using a solution of known concentration

To examine the controlled-release ability of the TA sheet *in vitro*, the sheet was placed in 2 mL phosphate-buffered saline (PBS) for 24 h and then placed in PBS containing 5 μg/mL collagenase D (Roche Diagnostics, Mannheim, Germany) for another 24 h. The release test was carried out at 37 °C and the PBS was exchanged at different time points. The supernatant was collected and freeze-dried, followed by dissolution in methanol. After centrifugation (8000×*g*, 10 min, 4 °C), the amount of TA in the supernatant was determined by HPLC.

2.3. Mouse skin wound model

Twenty-one C57BL/6J female mice (13 weeks old) were purchased from Asatsuma Dobutsu Kizaiten (Niigata, Japan). All animal experiments were conducted in accordance with the guidelines of Niigata University.

To prepare skin wounds, the mice were anesthetized with 0.1 mL/body weight (g) intraperitoneal anesthesia of 3% dexmedetomidine hydrochloride (DOMITOR®, Nihonzenyakukogyo, Fukushima, Japan), 8% midazolam (Dormicum®, Astellas, Tokyo, Japan), 10% butorphanol tartrate (Vetorphale®, Meiji Seika Pharma, Tokyo, Japan), and 79% distilled water. The dorsal hair was shaved with an electric clipper. An 8-mm punch biopsy tool (Maruho, Osaka, Japan) was used to make two skin wounds on the anterior side and posterior side on the back of the mice as shown in Fig. 1-a. The interval of the two skin wounds was more than 1 cm.

A 1-cm^2 (10×10 mm) piece of the TA sheet and control sheet was attached to the skin wounds of 11 mice (TA sheet on the head wound of 6 mice and on the tail wound of 5 mice) (Fig. 1-b), and the wounds were covered with an occlusive dressing (IV3000, Smith & Nephew, Tokyo, Japan) (Fig. 1-d). To determine the efficacy of the prepared TA sheet on the skin wounds, experiments comparing TA sheet versus TA injection were conducted using 10 mice (TA sheet on the head wound of 5 mice and on the tail wound of 5 mice). For the injections, we prepared a TA suspension containing 10 mg TA per 1 mL distilled water, and 0.1 mL (1 mg TA), which was the quantity of TA on the 1-cm^2 TA sheet, was injected on the edge of the skin wound (Fig. 1-c). Fifteen days after attaching the gelatin sheet, the mice were euthanized, and the tissues were analyzed as described below. The wound area was treated as an ellipse (Surface area (S) = πab) and compared with that of the original skin wound (15-day wound area/original skin wound area) (Fig. 1-e).

2.4. Histological and immunohistochemical analysis of mice

The tissues were fixed in 10% formaldehyde, embedded in paraffin blocks, and cut into 5-μm-thick sections on the middle line of the wound. The sections were deparaffinized with xylene, rehydrated with decreasing concentrations of ethanol, and then stained with hematoxylin and eosin (H&E). Sections for alpha smooth muscle actin (α-SMA) immunohistochemical staining were placed in citrate buffer at 100 °C for 15 min (antigen retrieval) and endogenous tissue peroxidases were inactivated with 0.3% H_2O_2 for 15 min. The sections were incubated overnight at 4 °C with rabbit monoclonal anti-α-SMA antibody (ab5694; Abcam, Cambridge, UK) at a dilution of 1:200. After washing with PBS for 5 min, the sections were incubated for 30 min at 25 °C with a VECTASTAIN ABC Kit (Vector Laboratories, Inc., Burlingame, CA, USA). After washing for 5 min with PBS, the sections were stained with 3,3′-diaminobenzidine (DAB) and hematoxylin.

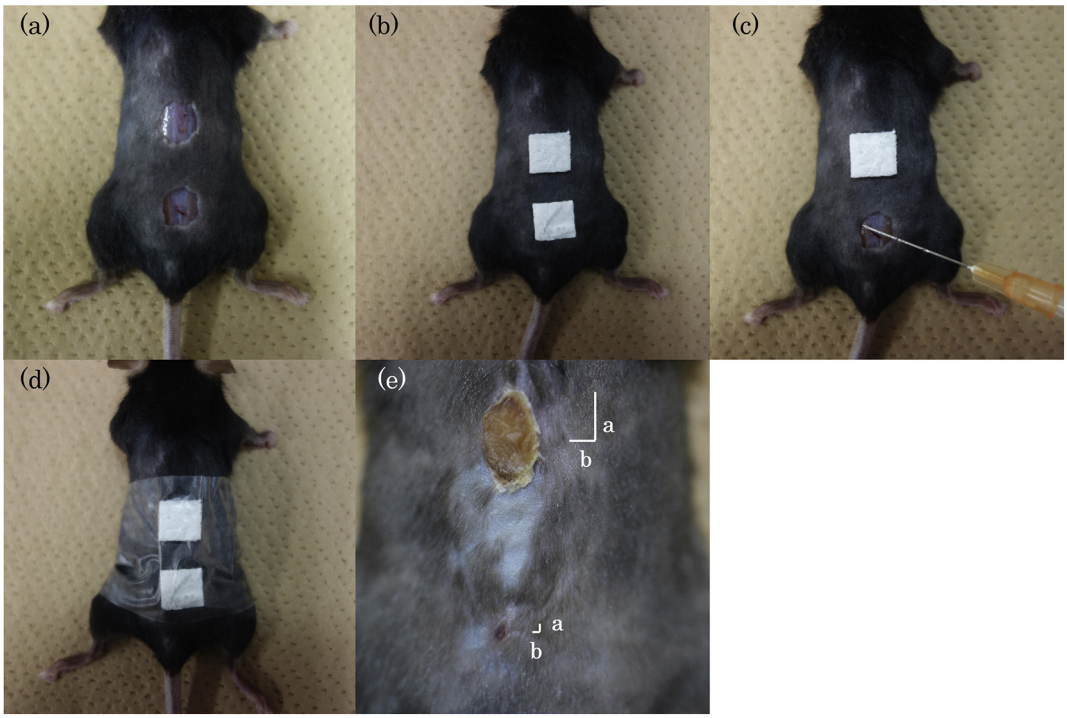

Fig. 1. Mouse skin wound model (a) Skin wounds were made with an 8-mm biopsy punch tool. (b) 10 × 10 mm TA sheet or control sheet was attached to each wound. (c) 10 × 10 mm TA sheet was applied or TA injection was conducted for each wound. (d) The wounds were covered with an occlusive dressing. (e) The wound area was considered an ellipse and its surface area compared with that of the original skin wound. The 15-day wound area/original skin wound area was evaluated.

H&E staining was conducted to assess the grade of epithelialization. We defined Grade 1 (slow) as epithelialized skin/original skin wound area <30%, Grade 2 (moderate) as epithelialized skin/original skin wound area from 30 to 70%, and Grade 3 (rapid) as epithelialized skin/original skin wound area >70% (shown in Fig. 2-a). The original skin wound was defined as a lack of superficial muscle layer.

To evaluate myofibroblast infiltration, α-SMA immunohistochemical staining (Fig. 2-b) under 100 × magnification was evaluated with ImageJ software (NIH, Bethesda, MD, USA). The α-SMA hot-spot area was selected and the proportion of α-SMA-positive area/whole area (%) was calculated (Fig. 2-c).

2.5. Statistical analysis

For macro and histological findings, continuous variables were expressed as the means ± SE and range and non-continuous data were expressed as percentages when there were more than three cases. Mann–Whitney U tests were used to compare the non-parametric data of macro findings and histological findings, with statistical significance set at $p < 0.05$. Statistical analysis was performed using SPSS Version 21 software (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Analysis of TA sheets

The concentrations of TA in different regions of the sheet were as follows: A, 0.832 mg/cm^2; B, 0.844 mg/cm^2; C, 0.998 mg/cm^2; and D, 0.936 mg/cm^2 (Fig. 3-a). Fig. 3-b shows the release profiles of TA from the sheet *in vitro*. In PBS, 7.7 ± 2.3% of TA was released from the sheet over 24 h. After the initial PBS change to PBS containing collagenase, the amount of released TA increased over time.

3.2. Histological and immunohistochemical analyses

3.2.1. Comparison of TA sheet with control sheet

3.2.1.1. Epithelialization. The wound area/original skin wound area (%) after 15 days with the TA sheet was significantly larger than that with the control sheet (26.9 ± 5.5% vs 10.7 ± 2.6%, $p = 0.023$) (Fig. 4-a).

The TA sheet wound was deemed Grade 1 in 5 mice (45.5%), Grade 2 in 1 mouse (9.1%), and Grade 3 in 5 mice (45.5%). In contrast, that of the control sheet wound was deemed Grade 1 in 2 mice (18.2%), Grade 2 in 0 mice (0%), and Grade 3 in 9 mice (81.8%) (Fig. 4-a).

3.2.1.2. Anti-fibrotic effect

The α-SMA positive area/whole area with the TA sheet was significantly smaller than that with the control sheet (4.65 ± 0.66% vs 7.24 ± 0.70%, $p = 0.023$) (Fig. 4-a).

3.2.2. Comparison of TA sheet with TA injection

3.2.2.1. Epithelialization. The wound area/original skin wound area after 15 days with the TA sheet (17.7 ± 4.5%) was larger than that with TA injection (11.1 ± 1.9%), but the difference was not significant ($p = 0.22$) (Fig. 4-b).

The TA sheet wound was deemed Grade 1 in 3 mice (30.0%), Grade 2 in 2 mice (20.0%), and Grade 3 in 5 mice (50.0%). The TA injection wound was deemed Grade 1 in 4 mice (40.0%), Grade 2 in 4 mice (40.0%), and Grade 3 in 3 mice (30.0%) (Fig. 4-b).

3.2.2.2. Anti-fibrotic effect

The α-SMA positive area/whole area with the TA sheet was significantly smaller than that with the TA injection (5.32 ± 0.45% vs 7.93 ± 0.75%, $p = 0.013$) (Fig. 4-b).

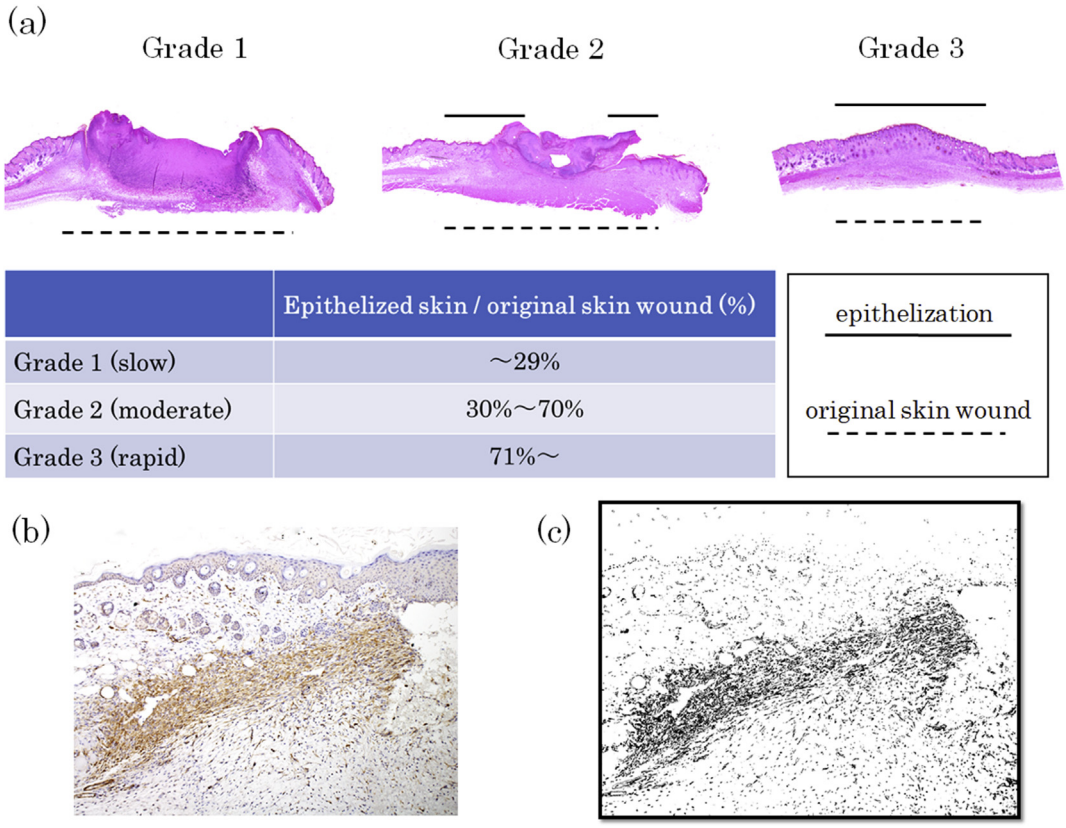

Fig. 2. Histological and immunohistochemical analysis of mice (a) Hematoxylin and eosin (H&E) staining was used to access the grade of epithelialization. Grades 1–3 were defined as described in section 2.4. (b) Immunohistochemical staining for alpha smooth muscle actin (α-SMA) was also performed to access myofibroblast infiltration at 10×10 magnification. (c) The image was processed by ImageJ and the positive area (black area) was calculated at 10×10 magnification.

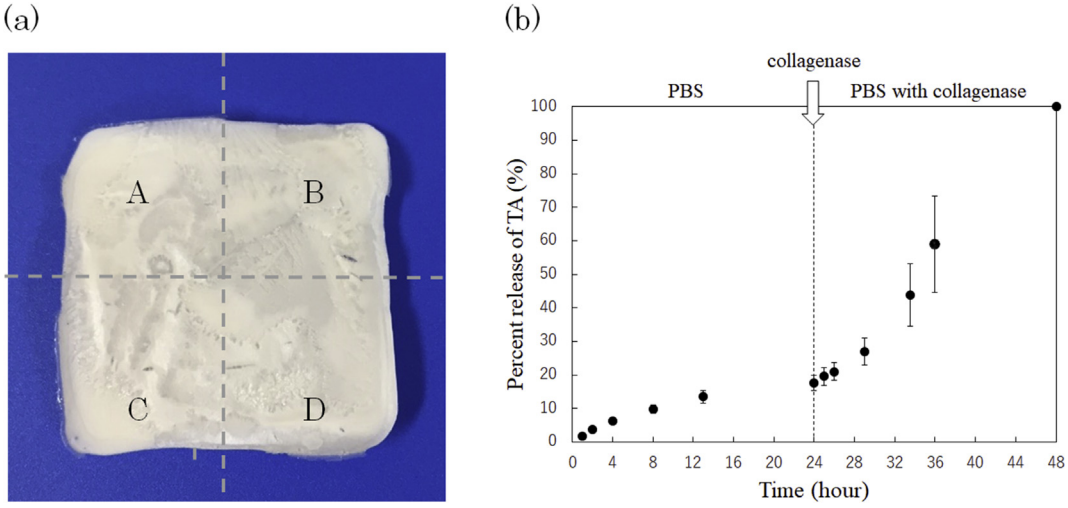

Fig. 3. Analysis of TA sheets (a) Photograph of the TA sheet. To evaluate the homogeneity and quantity of TA in the sheet, high-performance liquid chromatography (HPLC) analysis was performed. Quantity of TA in different regions was A: 0.832 mg/cm^2, B: 0.844 mg/cm^2, C: 0.998 mg/cm^2, and D: 0.936 mg/cm^2. (b) *In vitro* release profiles of TA (black dots) from gelatin hydrogels. The release test was carried out at 37 °C and PBS was exchanged at different times. The sheet was placed in PBS without collagenase for 24 h, followed by PBS containing collagenase for another 24 h.

4. Discussion

In this study, we prepared a TA suspension gelatin sheet (TA sheet) and confirmed both the homogeneity of suspended TA and controlled-release of TA in the presence of collagenase *in vitro*. The TA sheet decreased the rate of skin wound regeneration and caused less myofibroblast infiltration into the tissue than the control sheet did. In addition, the TA sheet caused less myofibroblast infiltration into the tissue than TA injection.

In this study, we adopted a mouse skin wound model to investigate epithelization and fibrosis. Sakai et al. reported the efficacy of a pioglitazone gelatin-sheet using a mouse skin wound model [5]. Based on this previous study, we evaluated epithelialization by both the macroscopic wound area and histological

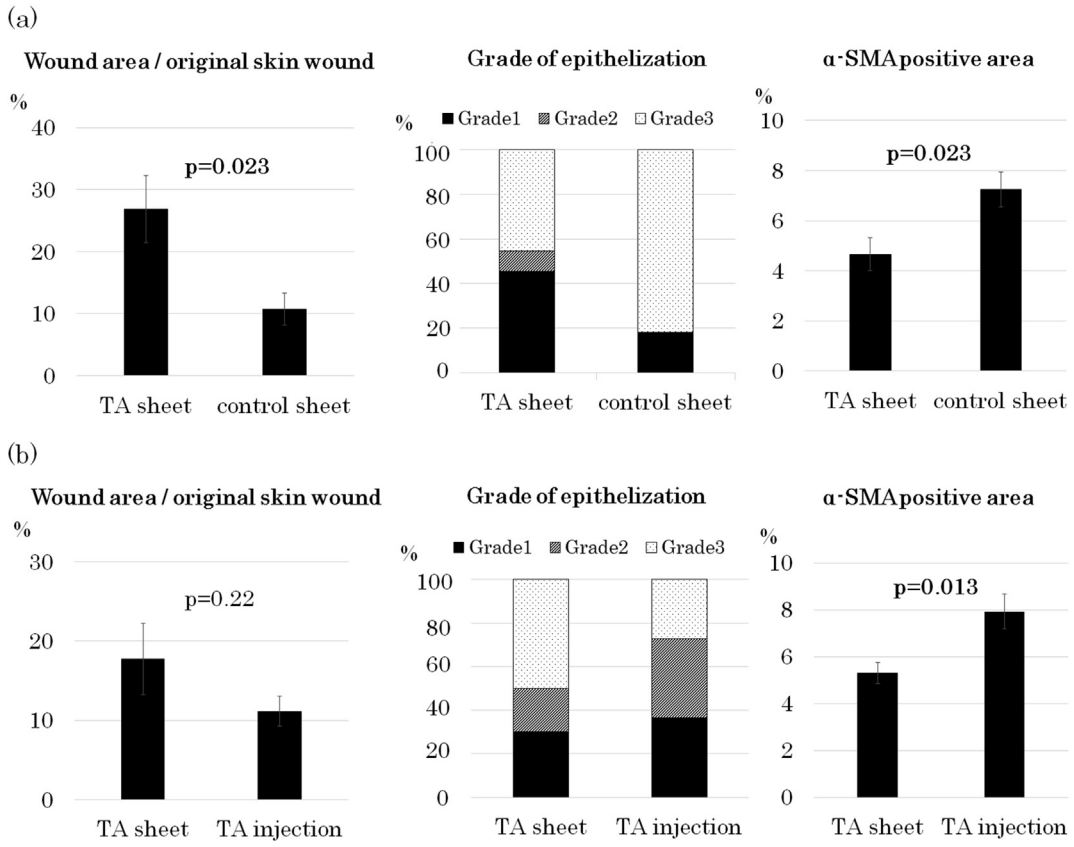

Fig. 4. Histological and immunohistochemical analyses (a) Comparison of triamcinolone acetonide (TA) sheet with control sheet. Wound area/original skin wound (%), grade of epithelialization, and α-SMA-positive area (%) were evaluated. (b) Comparison of TA sheet with TA injection. Wound area/original skin wound (%), grade of epithelialization, and α-SMA-positive area (%) were evaluated.

degree of epithelial regeneration. To determine the efficacy of the prepared TA sheets, a comparison to TA injection was utilized because TA injection is used clinically to prevent inflammation and fibrosis [8,9]. To assess myofibroblast infiltration, we conducted α-SMA immunohistochemical staining, which is widely used [10]. Additionally, we used ImageJ software for quantitative histological assessment, as previously reported [11].

Scar formation is thought to be an integral part of wound healing, a process that involves inflammation, proliferation, and remodeling. Collagen is the major fibrous connective tissue protein and provides structural support in scars [12]. Corticosteroids decrease collagen and glycosaminoglycan synthesis by reducing the inflammatory process in the wound, decreasing fibroblast proliferation, and increasing hypoxia [13]. Corticosteroids are potent vasoconstrictors that reduce the delivery of oxygen and nutrients; they lead to a decrease in production of inflammatory cytokines such as vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), and interleukin-1 (IL-1) [14]. Collectively, these mechanisms result in the suppression of wound healing TA, a corticosteroid, is often used in clinical situations in which fibrosis causes dysfunction. For example, for linear hypertrophic scars arising from surgery or trauma, recent expert guidelines have advised surgical resection with adjuvant intralesional TA therapy to reduce the risk of skin contracture formation [15,16]. Hashimoto et al. reported that TA injection into esophageal mucosal defects was effective for the prevention of esophageal stricture formation after endoscopic treatment for superficial esophageal cancer [17]. Pal et al. reported that absorbable gelatin sponges soaked in TA are effective for the treatment of temporomandibular joint ankylosis [18]. We adopted TA as our steroid preparation because it has been in widespread clinical use.

Gelatin is a biodegradable material that has been used extensively for food, pharmaceutical, and medical applications. It has been shown to be an effective carrier for drug release and can augment the therapeutic activities of both water-soluble [7,19–21] and water-insoluble drugs [5,6,22], such as pioglitazone for wound healing [5], simvastatin for bone regeneration [6], and neuropeptide substance P for angiogenesis [7]. Water-insoluble drugs can be delivered to tissues by using polymeric micelles and through their controlled release [6]. Typically, these drugs cannot remain in the wound area after direct application. Thus, controlled release is important and can enhance drug activity. The use of gelatin as a drug-release matrix has two advantages compared to other release systems. First, gelatin is preferred to suppress material-induced inflammatory responses [23]. Second, gelatin can release the incorporated drug by its degradation, and it is eventually eliminated. For these reasons, we adopted gelatin as a drug-release matrix in our study.

To the best of our knowledge, there have been no reports on TA-loaded gelatin to treat wound healing *in vivo*. An important study on TA-soaked gelatin sponges for the treatment of temporomandibular joint ankylosis [18] was reported. However, a detailed description of the gelatin sponges, such as their formulation and sustained-release of TA, was not provided in the paper. Although Hamishehkar et al. reported an optimized oral paste formulation of TA to be used to treat aphthous stomatitis [24], the formulation was only tested in *in vitro* experiments. Their mixture of hydrocolloid

solids included gelatin, pectin, and sodium carboxymethylcellulose. Our study is the first report on a TA-loaded gelatin sheet in a mouse skin wound model. We produced a novel TA sheet by suspending water-insoluble TA into the sheet and we verified its effect both *in vitro* and *in vivo*. Furthermore, compared to previously reported methods, our method is very simple and requires fewer procedures and less preparation time. The TA sheet was uniform and showed sustained release in this study.

The TA sheet caused less myofibroblast infiltration into the tissue than TA injection. The reason for the superior properties of the TA sheet compared to those of TA injection may be that the gelatin sheet exhibits more uniform and sustained TA release. Although the gelatin sheet may be suitable for preventing fibrosis, epithelization was also relatively suppressed with TA injection compared to that using the TA sheet. Although it is speculation, we believe that injected TA penetrates the subepithelial tissue nonuniformly and deeply from the center of the puncture site. In contrast, the sustained-release TA penetrates the subepithelial tissue uniformly and slowly from the sheet on the surface of the skin wound. We believe that this difference in penetration of TA into the tissue affects the epithelization of mucosal wounds.

Using the TA sheet, we achieved positive results suppressing epithelization and preventing fibrosis. However, many investigators have attempted to accelerate wound closure using several dressing materials Is the TA sheet beneficial for the treatment of wound healing? We believe that it has potential to be effective for the prevention of fibrosis during healing of local and small wounds and lesions. This includes attenuating strong skin contracture due to burn injury, joint ankylosis, and esophageal cautery ulcers due to endoscopic treatment, among others. The purpose of using TA sheets is to suppress severe fibrosis to allow epithelial cells to regenerate.

The TA sheet may have some limitations. Because the sheet does not show strong adhesive characteristics, it is slightly difficult to prevent it from moving when placed on a skin wound. Therefore, the sheet must be rigidly fixed to the skin. For clinical use in an unstable environment, such as in the gastrointestinal tract, the sheet delivery and anchoring method must be improved. In these situations, it will be necessary to develop improved TA sheets that have superior fixing strength.

5. Conclusions

In conclusion, we produced a TA sheet and evaluated its efficacy in preventing myofibroblast infiltration into skin wounds of the mouse dorsa. Thus, various clinical situations in which local fibrosis causes dysfunction, such as those involving the skin or gastrointestinal tract, can be treated with this TA sheet.

Declarations of interest

Nothing declared.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2019.04.001.

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