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Accelerating Effects of Silk Fibroin on Wound Healing in Hairless Descendants of Mexican Hairless Dogs

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Abstract: We examined the safety of silk fibroin in the subcutaneous tissues. In addition, we macroscopically and histopathologically evaluated its healing effect on the full-thickness wounds in hairless dogs. We prepared 3 types of matrices including amorphous silk fibroin films, and α-and β-type silk fibroin powder. No toxicity was found in the sites injected with silk fibroin solutions. Macroscopically, silk fibroin films had accelerating effects on wound repair, as compared with occlusive dressings (dried porcine skin and hydrocolloid dressings). Histopathological examinations revealed that silk fibroin films facilitate reepithelialization and the formation of granulation tissues, collagens and elastic fibers. The sites treated with the α-type silk fibroin powder were faster in wound repair than those treated with the β-type silk fibroin powder. The α-type silk fibroin powder absorbed excessive exudate. Microscopically, there are epidermal and dermal regeneration in the sites treated with the α-type silk fibroin powder. These results show that silk fibroin is inert in biological tissues, indicating excellent biocompatibility. Silk fibroin films facilitate reepithelialization, remodeling of connective tissues and collagenization. The α-type fibroin powder is a wound dressing that regulates excessive exudate from the wound and provides a proper moist environment. These results suggest that silk fibroin is a useful dressing material in veterinary clinical medicine.

Keywords: hairless dogs, silk fibroin, wound dressings, wound healing

INTRODUCTION

Native silkworm silk fibers from *Bombyx mori* consist of a core structural protein fibroin that is coated with sericin, a family of glue-like proteins that hold the fibroin core fibers together.[1] These silk fibers have been used for decades as sutures in biomedical application[2] and have a potential as scaffolds in tissue engineering.[3,5]

Since Tsubouchi[6] and Tsubouchi *et al.*[7] reported that silk fibroin was effective in repairing skin injury, interest has been increasing in the use of solubilized silk fibroin in veterinary dermatological science. On the basis of experience thus far, wound dressings for human plastic surgery have been applied to treatment of wounds in small animals.[8,13] In veterinary medicine, there is a paucity of animal experimentation using silk fibroin.[14,17]

Recently, we have established a colony of hairless descendants of Mexican hairless dogs.[18] Hairless dogs have been utilized for investigating photodermatology,[19,22] delayed contact hypersensitivity,[23,26] age-related changes in the skin[27,29] and plastic surgery[30,32].

In the present study, we prepared 3 types of matrices including amorphous silk fibroin films, the α-type silk fibroin powder (the distorted conformation, random coil or silk α) and the β-type silk fibroin powder (the more stable β-sheet or silk β). Then, I applied 3 types of matrices to the full-thickness wounds in hairless dogs.

The purpose of this study on wound healing was to threefold: first, to assess the safety of silk fibroin in subcutaneous tissues, second, to compare silk fibroin films with commercial occlusive dressings, and third, to evaluate the differences in wound healing between the α- and β- type silk fibroin powder.

MATERIALS AND METHODS

Experiment 1:

Animals: 10 female HR-1 hairless mice were purchased at 5 weeks old from Japan SLC Co., Ltd. (Shizuoka, Japan). The mice were acclimatized for 1

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week before use. They were housed 5 animals/plastic cage in an air-conditioned room (temperature, 25 ± 1 °C; relative humidity, 50 ± 10 %) with a 12-hr light/dark cycle. Diet (CE-2, Clea Japan, Inc., Tokyo, Japan) and tap water were available *ad libitum*.

Procedure: Silk fibroin films were dissolved in saline, and then 10 % silk fibroin solution was subcutaneously injected in both sides of the dorsal skin of 5 hairless mice. The dose of silk fibroin solution was 0.2 ml per site. Five another mice administrated 0.2 ml of saline served as a control.

Macroscopic observations were done before the study and daily during the experiment. The right side of each mouse was biopsied 3 days after the subcutaneous injection of 10 % silk fibroin solution. Likewise, another side was biopsied 7 days after this procedure.

Experiment 2:

Dogs: A female dog was used in this study. The dog was N5 hairless hybrids resulting from the breeding of male N4 hairless dogs and female beagle dogs.

The dog was individually housed in a stainless steel cage (90 × 90 × 90 cm) in an animal room controlled at 25 ± 1 °C and 50 ± 10 % relative humidity with 10 to 15 exchanges of 100 % fresh air/hour and 12-hour light (7AM to 7PM), 12-hour dark (7PM to 7AM) cycle. The dog was fed a commercial dry dog food (TC-2, Aixia Corporation, Tokyo, Japan) and water *ad libitum*.

Wound Dressing (WD): The following reagents and dressing materials were tested in this study: 1. WD-untreated (only bandage, control), 2. Silk fibroin films, 3. dried porcine skin (DP skin, Alloask D, Taiho Yakuhin Co., Ltd., Tokyo, Japan), 4. Hydrocolloid dressings (DuoActive Brystol Myers Squibb, Co., Ltd., USA). Four split thickness wounds were made on the dorsal site of the animal.

Procedures: The animal was anesthetized with ketamine hydrochloride (Ketalar, Sankyo Co., Ltd., Tokyo, Japan) after medetomidine (Domitol, Meiji Seiyaku Co., Ltd., Tokyo, Japan) premedication. Split thickness wounds (1 × 1 cm) were made on the dorsal skin of the dog, using sterile surgical blades (Feather Safety Razor Co., Ltd., Osaka, Japan). The blade of a scalp was used to dissect the epidermis and the dermis over the whole wound area. After irrigating the wound with sterile physiological saline and 0.05 % chlorhexidine gluconate (Hibitane, Sumitomo Seiyaku, Co. Ltd., Osaka, Japan), each dressing material was applied to the split thickness wound at a rate of approximately 10 mg/cm^2 (Fig. 1). The WD-treated sites were covered with transparent adhesive polyurethane film (Tegaderm, 3M Health Care, Tokyo, Japan). Canine protective jackets and Elizabethan collars (Tsugawa Yoko Co., Ltd., Tokyo, Japan) were used to prevent the dog from dislodging the dressings or from peeling off. The WD-treated sites were kept under the wet environment during this study. Keeping wounds moist allows normal epidermal migration at the same level as the undamaged epidermis and without shrinkage. The dressings were changed on day 7 of the study. The adhesive polyurethane film was also changed on this day.

Clinical Evaluations: Clinical observations were done before the study and daily during the WD-treatment. The WD-treated sites were evaluated daily through the transparent adhesive film, and clinically observable changes (reepithelialization, granulation, inflammatory reactions and exudation) were photographed.

Histopathological Examination: Tissue specimens were obtained from both WD-treated and untreated sites of the dorsal skin of the dog using a 6-mm biopsy punch under general anesthesia with medetomidine on the day before the study, and at 7 and 14 days after the study start. Skin biopsy was carried out as follows: the right side at 7 days and the left side at 14 days after the study. Skin specimens were fixed in 10 % neutral buffered formalin, and 4-μm paraffin sections were stained with hematoxylin and eosin (HE) and toluidine blue (TB), and by van Gieson's (vG) and Weigert's (WG) staining.

Silk Fibroin Films: Raw silk produced by *Bombyx mori* silkworms was degummed twice with 0.5 % aqueous Na_2CO_3 at 100 °C for 1hour and washed thoroughly with distilled water. Degummed silk was dissolved in a mixed solvent of $CaCl_2$, H_2O and ethanol with a 1 : 8 : 2 molar ratio at 80 °C for 6 hours. The silk fibroin solution was autoclaved after dialysis with cellulose tubular membrane in distilled water for 3 days. The silk fibroin films were prepared by casting the above-mentioned solution onto a polystyrene plate and allowing the solvent to evaporate at room temperature.

Silk Fibroin Powder: Aforementioned silk fibroin films were ground into the α-type silk fibroin powder. Additionally, silk fibroin films dealt with ethanol were ground into the β-type silk fibroin powder.

Experiment 3:

Dogs: Another female dog was used in this study. The dog was N5 hairless hybrids resulting from the breeding of male N4 hairless dogs and female beagle dogs. This dog was kept in the same manner as described in Experiment 2.

Procedures: Test procedures were the same way as performed in Experiment 2. WD-material (the α- or β-type silk fibroin powder) was applied to the split thickness wound at a rate of approximately 10 mg/cm^2. Untreated sites served as controls.

Clinical Evaluations: Clinical observations were done before the study and daily during the WD-treatment.

Histopathological Examination: Tissue specimens were obtained on the day before the study and 7 days after the study start. Histopathological examinations were the same as aforementioned method.

RESULTS AND DISCUSSIONS

Experiment 1: The toxicity of silk fibroin was tested. Macroscopically, no visible abnormalities were found in the skin injected with silk fibroin solutions and saline throughout the study. There were few microscopical changes in the test sites 3 and 7 days after the subcutaneous injection of silk fibroin solution (Fig. 1).

Experiment 2:

Gross Appearances: Seven days after the beginning of this study, the WD-untreated sites remained unepithelialized. The wound surface was covered with a thick crust.

The sites treated with silk fibroin films showed suitable formation of healthy granulation tissues. Reepithelialization had begun from the rim of the normal skin, and wound healing had attained more than half of the wound surface (Fig. 2-A).

The sites treated with DP skin exhibited profound adherence of the wound edge to the overlying dressing. DP skin inhibited reepithelialization and granulation tissue formation. The wounds treated with DP skin contracted as compared with those treated with the other WDs (Fig. 2-B).

The exudate underneath DP skin was tenacious and difficult to remove from the intact skin, whereas the exudate underneath HC dressing was easier to remove from the around skin. HC dressings were hydrophilic and absorbed excessive wound exudates. HC dressings provided an environment to keep wounds clean and moist, and the materials enhanced early reepithelialization and granulation (Fig. 2-C).

Fourteen days after the beginning of this study, the center of the WD-untreated sites remained unepithelialized, while the sites treated with silk fibroin films had almost completely repaired.

The treatment with DP skin delayed wound healing in the skin of hairless dog, as compared with the WD-untreated sites. In the sites treated with DP skin,

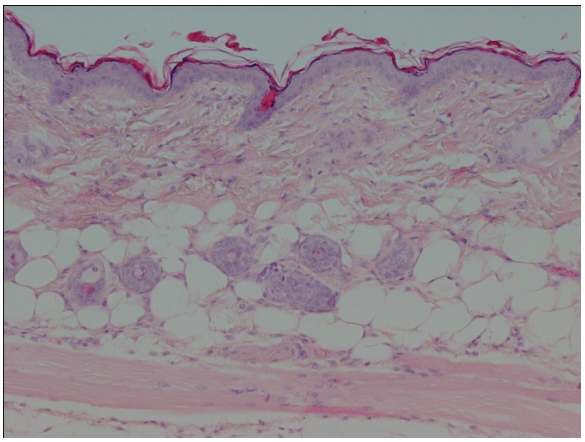

Fig. 1: Histopathological photograph 7 days after the subcutaneous injection of silk fibroin solution. No histopathological changes are seen. HE stain, × 100.

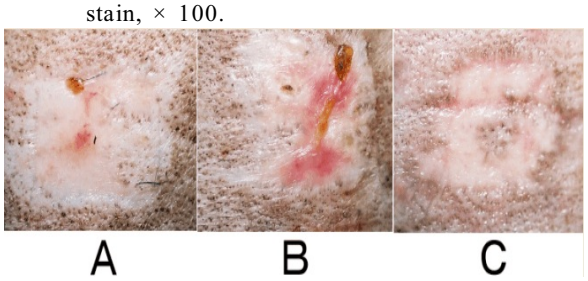

Fig. 2: Macroscopic photographs 14 days after treatment.
A. The site treated with a silk fibroin film.
B. The site treated with PD skin.
C. The site treated with HC dressing.

neither reepithelialization nor granulation was sufficiently completed by 14 days after treatment. Exudate formation and moderate inflammatory reactions were observed.

The sites treated with HC dressings had completely reconstructed and the reepithelized skin developed repigmentation.

Histopathological Findings: Seven days after the beginning of this study, the WD-untreated sites were not fully epithelialized and the neodermis was not reconstructed. Under the crust, reepithelialization gradually began from the edge of the intact skin. Epithelial sliding was present in the WD-untreated sites. Although inflammatory granulation tissues were formed, the neodermis was not reconstructed. The formation of elastic and collagen fibers was delayed. Thick and flat epidermis was observed in the sites treated with silk fibroin films. Keratinization of the new epithelium was present and mitotic figures were

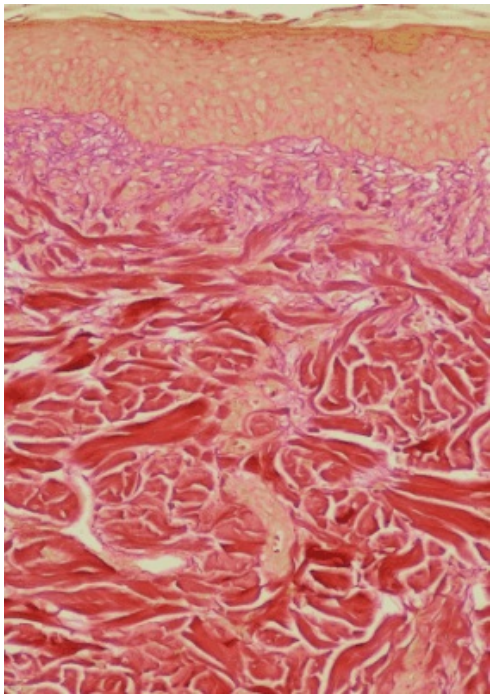

Fig. 3: Histopathological photograph 7 days after treatment with a silk fibroin film. Reepithelialization and the formation of collagen fibers in the dermis. vG stain, × 100.

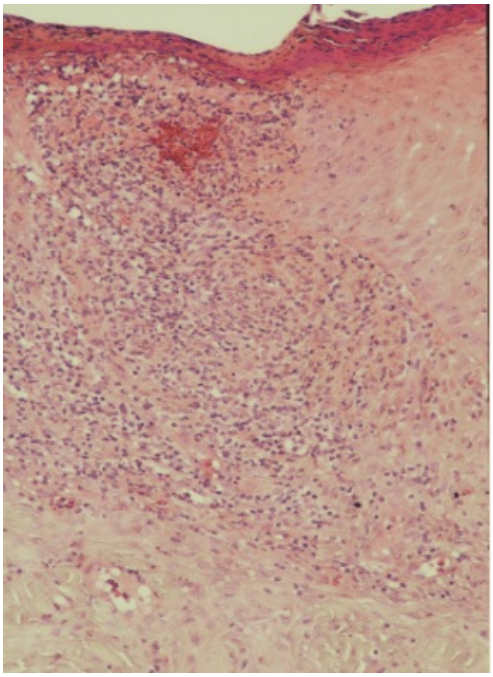

Fig. 4: Histopathological photograph 7 days after treatment with PD skin. Thick crust and severe inflammation and hemorrhage are seen. Reepithelialization is inhibited. HE stain, × 100.

noted in the advancing epithelium. There was apparently less edema in the wounds. Infiltration of mononuclear inflammatory cells was also slight. Reepithelialization and granulation tissue formation were observed at the edge of the intact skin tissue. Fine collagen and elastic fibers were observed in the dermis (Fig. 3).

The sites treated with DP skin were apparently edematous and covered with a thick crust. Severe infiltration of mononuclear inflammatory cells was found in the subcutis of the wound. Both reepithelialization and granulation tissue formation were disturbed under the scab. A great number of inflammatory cells and marked hemorrhage inhibited the formation of collagen and elastic fibers (Fig. 4).

In contrast, there was apparently less edema in the sites treated with HC dressings than those treated with the other dressings. Reepithelialization and thick epidermal cells grew over the surface of the wound from the rim of the intact skin. Well-developed papillary layers were also found. Abundant collagen bundles and fibroblasts arranged parallel to the epidermal-dermal junction. This configuration implied organized reconstruction of dermis without excessive scar formation (Fig. 5).

Fourteen days after the beginning of this study, the WD-untreated sites were fully reepithelialized and a large number of microvessels developed underneath the epidermis. Edematous and hemorrhagic lesions still remained. Collagen and elastic fibers were not fully constituted.

In the sites treated with silk fibroin films, regenerated epidermis completely covered the wound surface. Mononuclear inflammatory cells were disappearing in the dermis. Granulation tissues started to be replaced by collagen and elastic fibers (Fig. 6).

Although the sites treated with DP skin almost reepithelialized, inflammatory cell infiltration still remained in the granulation tissues. Moderate edema (vacuoles) developed in the epidermal-dermal junction. In the sites treated with HC dressings the epidermis recovered its normal architecture and differentiation of surface cells yielded a mature structure with surface keratinization. In addition, there were melanin pigmentation and some melanocytes with dendrites in the *stratum basales.* Dense collagen and fine elastic fibers with scattered vascular channels replaced granulation tissues.

Macroscopically and microscopically, regeneration of the skin around the wounds was faster in the sites treated with silk fibroin films and HC dressings than in the WD-untreated sites. Wound healing was accelerated in the order of HC dressing > silk fibroin films > WD-untreated > DP skin.

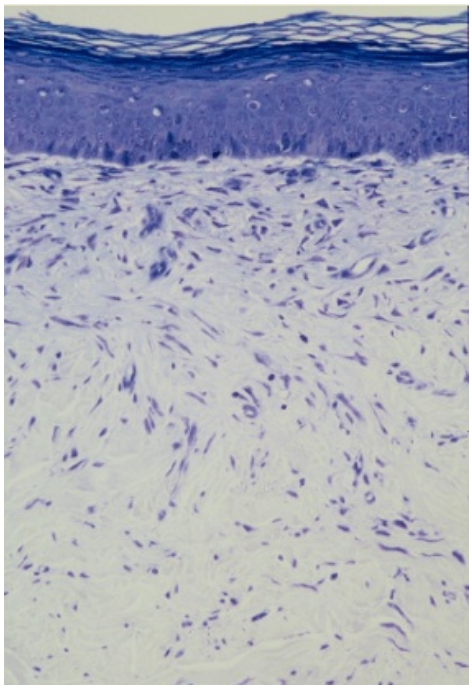

Fig. 5: Histopathological photograph 7 days after treatment with HC dressing. Reepithelialization and granulation tissue formation are seen. vG stain, × 100.

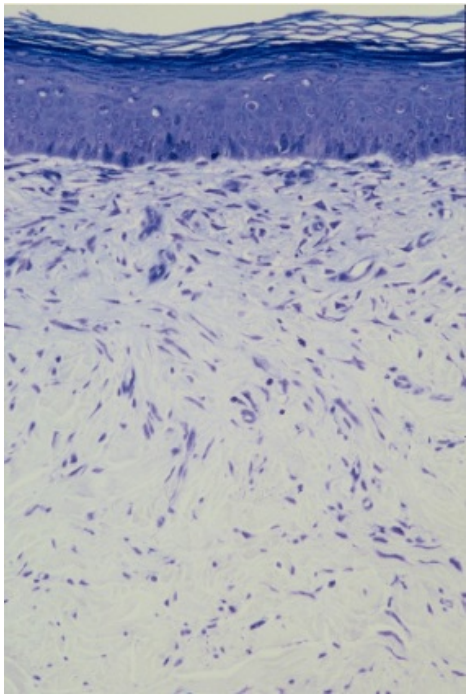

Fig. 6: Histopathological photograph 14 days after treatment with a silk fibroin film. Regeneration of the epidermis and the dermis are seen. TB stain, × 100.

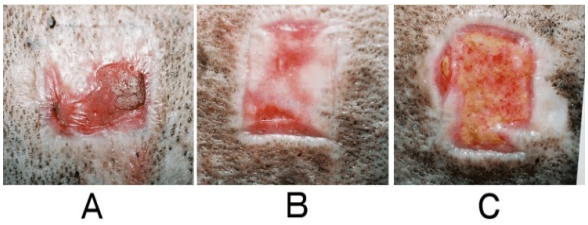

Fig. 7: Macroscopic photographs after treatment.
A. The WD-untreated site.
B. The site treated with α- type fibroin powder.
C. The site treated with β- type fibroin powder.

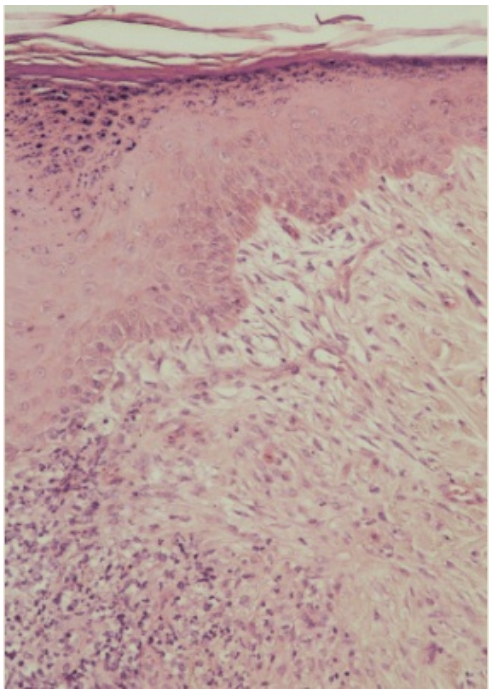

Fig. 8: Histopathological photograph after treatment with α-type fibroin powder. Thick reepithelialization and granulation tissue formation are seen. HE stain, × 100.

Experiment 3:

Gross Appearances: There were notable differences in the amount of exudate among the sites treated with 3 kinds of dressings. Seven days after the beginning of the study, the WD-untreared sites showed abundant exudate including hemorrhage. In addition, wound contraction resulted from a scab forming on top. There was minimal fluid production under the α-type fibroin powder. The β-type fibroin powder had moderate fluid production at all positions. As the wounds matured under the dressings, the fluid production was less. In the early stage of the wound healing, the α-type fibroin powder allowed more absorption of exudate than the β-type fibroin powder.

Reepithelialization was delayed in the WD-untreated sites. The α-type fibroin powder promoted wound healing by facilitating reepithelialization. The β-type fibroin powder adhered the wound surface, resulting in incomplete reepithelialization. The edge of the wound appeared as some swelling on the skin. It appeared that the α-type fibroin powder was the best treatment evaluated in the Experiment 3.

Granulation tissues were present in all wounds 7 days after this study. However, exuberant granulation tissues were evident on the sites treated with the α-type fibroin powder (Fig. 7).

Microscopic Findings: Seven days after the beginning of this study, the WD-untreated sites were incompletely reepithelialized and these sites were covered with a thick crust. There were marked infiltration of mononuclear inflammatory cells and fibroblasts and severe hemorrhage. There was deficient regeneration of collagen and elastic fibers in the dermis.

The sites treated with the α-type fibroin powder showed thick epidermis composed of hyperplastic keratinocytes (Fig. 8). Exuberant granulation tissues were observed and some areas of these tissues started to be replaced by fine collagen fibers (Fig. 9).

The sites treated with the β-type fibroin powder exhibited thick epidermis with epidermal ingrowths (Fig. 10). In the dermis, granulation tissues contained reconstructed microvessels filled with erythrocytes. Collagen and elastic fibers were not found in the dermis (Fig. 11).

Macroscopically and microscopically, wound healing was accelerated in the order of α-type fibroin powder > β-type fibroin powder > WD-untreated.

Discussion: The effects of silk fibroin on the wounds were clinically and histopathologically investigated in the dorsal skin of hairless dogs. The results in Experiment 1 revealed that silk fibroin did not have any toxic and irritant effects on the wound healing. Silk fibers are known to be unable to induce sensitization responses in human beings.[33] In addition, a recent study demonstrated that silk fibers inhibited the inflammatory response to a secondary stimulus such as infection in the area of a silk-based implant.[34] There is evidence to indicate that silk fibroin had no significant inflammatory properties.[15,17,35] It was probable that silk fibroin was immediately absorbed in the tissues around the injection sites.

In Experiment 2, the treatment with silk fibroin films proved to be favorable for wound healing in the hairless dog. Macroscopically, silk fibroin films were converted into thin transparent gel-like films over the treated wounds. Amorphous silk fibroin films gradually crystallized to water-insoluble α-type silk fibroin.[6] Silk

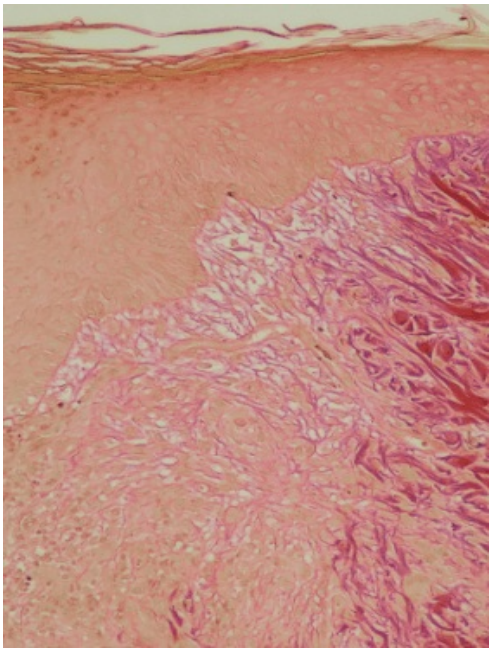

Fig. 9: Histopathological photograph after treatment withα- type fibroin powder.
Fine collagen fibers are seen. vG stain, × 100.

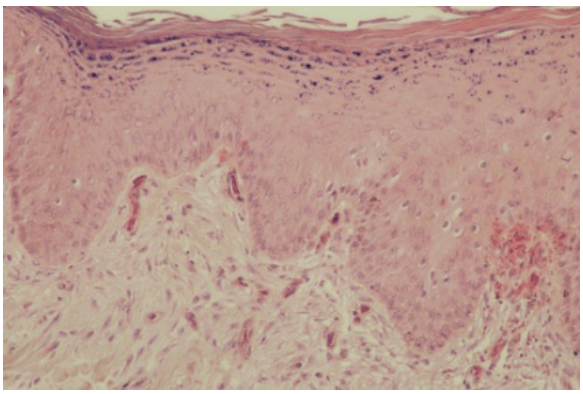

Fig. 10: Histopathological photograph after treatment with β-type fibroin powder. Reepithelialization is not fully noted. Hemorrhage is seen in the epidermal-dermal junction. HE stain, × 100.

fibroin films provided a moist environment for wound healing and these films accelerated early reepithelialization and granulation tissue formation, as compared with the WD-untreated sites. It is likely that this converting process of silk fibroin films have some associations with the acceleration of wound healing. The present results showed that the effects of silk fibroin films on reepithelialization developed from 7 to 14 days after treatment. Our findings accorded with those reported in therapeutic trials using silk fibroin films.[14]

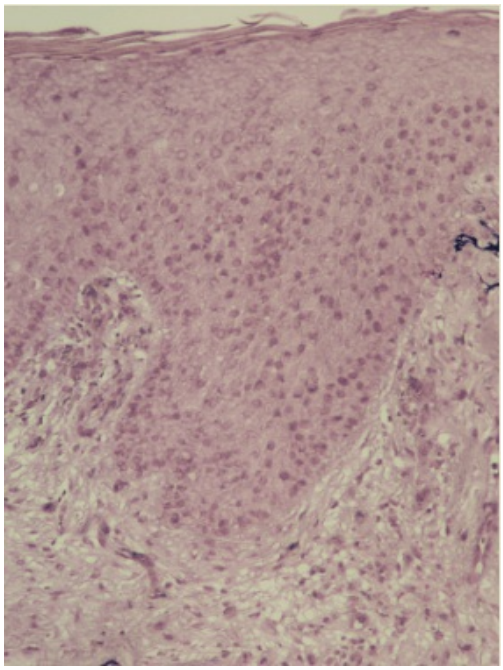

Fig. 11: Histopathological photograph after treatment with β-type fibroin powder. Granulation tissue formation and elastic fibers are not fully noted. WG stain, × 100.

Wound healing was markedly delayed in the sites treated with PD skin. These adverse effects seemed to be associated with immune reactions to a different species of protein. We should carefully treat biological dressings derived from other species.

HC served as an effective dressing for the full thickness skin wound. This result was close agreement with that of our previous report.[32] A moist wound environment is known to enhance reepithelialization and granulation tissue formation.[36] HC dressings absorbed excessive exudate and created an appropriate moist environment. Topical application of HD dressings did not affect wound contraction. Additionally, the hydrophilic properties of HD dressings may play some roles in wound healing.

Histopathological examinations revealed that reepithelialization and granulation tissue formation have been almost completed in the sites treated with silk fibroin films by 7 days after the beginning of this experiment. Subsequently, wound-healing effects of these dressings could be improved by concurrent increase in the production of collagen and elastic fibers. Histopathological findings in the repaired tissues showed that regeneration of parenchymal cells and remodeling of connective tissues have already started. These results revealed that wound healing reached at the advanced stage, collagenization by 14 days after the beginning of this experiment.

Throughout the present study, DP skin caused severe inflammatory reactions. Sugihara *et al.*[14] reported that the full-thickness wound treated with DP skin completely healed without edematous and inflammatory changes. They mentioned that the wounds treated with DP skin showed almost the same results as with silk fibroin films. These conflicting results revealed that there was a species difference in tissue reaction to DP skin.

Occlusive dressings are currently used to treat wounds in humans. These dressings prevent absorption of fluid from the surface of the wounds. Occlusive dressings such as HC dressings provide a moist environment. Occlusive dressings increase the rate of reepithelialization and dermal repair.[36] Wound fluid contains substances that increase the rate of wound healing and dressings should not be removed from the wound surface.[37] Our histological findings in the sites treated with HC dressings lent support to these previous observations. We confirm that proper management and debrigement of exuberant granulation tissues in the wounds treated with HC dressings enhance canine wound healing.

Experiment 3 exhibited that wound healing of the full-thickness skin defects treated with the α-type fibroin powder was faster than that of the untreated sites. The α-type fibroin powder had a property of absorbing readily excessive exudate and this substance provided a favorable moist environment for wound healing. The effects of the α-type fibroin powder on the wounds were similar to those of silk fibroin films observed in Experiment 2. As reported in our previous paper, the wounds in hairless dogs healed without apparent contraction.[32] This healing process was similar to that observed in human beings.

The α-type fibroin differed in water retention from the β-type fibroin. Promotive effects of silk fibroin on wound repair seemed to be attributable to the difference in the crystal structure between the α- and β-type fibroin.

Histopathologically, the reconstruction of the epidermis and the dermis was delayed in the WD-untreated sites, and inflammatory reactions still remained in the lesions. These findings agreed with those obtained in Experiment 2.

The α- type fibroin powder provoked prominent proliferation of the epidermal cells and the treated sites were fully epithlialized. Granulation tissues formation and collagenization followed reepithelialization. *In vitro* studies showed that silk fibroin supported the adhesion and spreading of cultured fibroblasts and keratinocytes.[4] This potentiality to promote cell growth has been applied to scaffolds for tissue engineering.[3,5] Our study using laboratory animals provided ample experimental support for above-mentioned *in vitro* tests.

Conformational changes of silk fibroin from random coil to β-sheet structure seemed to accelerate wound healing. In addition, Yamada *et al*.[38] have recently identified 2 kinds of fibroin-derived peptides as cell growth factors. These peptides may also have some associations with the enhancing effects of silk fibroin on wound healing.

Tsubouchi[6] reported that the β-type fibroin had a low affinity for human skin fibroblasts. It is probable that this property is related to our therapeutic results in the sites treated with the β-type fibroin powder.

Silk fibroin has suitable characteristics of wound covering materials. The shape and formulation of silk fibroin are important for its wound healing effects. Silk fibroin is expected to be clinically useful for veterinary wound management. The process of wound healing in hairless dogs is closely akin to that in human beings.[32] The present data may available to evaluate the effects of silk fibroin dressings on human wound treatment. Haired dogs are faster in wound repair than hairless dogs.[32] Silk fibroin seemed to have more promotive effects on veterinary wound treatment.

From 3 kinds of experiments, it was concluded that silk fibroin was inert in biological tissues, indicating excellent biocompatibility. Silk fibroin films facilitate reepithelialization, remodeling of connective tissues and collagenization. The α-type fibroin powder is a wound dressing that regulated excessive exudate from the wound. These results suggest that silk fibroin has a potential to become a dressing material that is equal to occlusive dressings.

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