Studies on PVA/Chitosan/Fibroin Blend Sponge Sheets: Preparation and Wound Healing Effects in Rats

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The preparation of PVA/Chitosan/Fibroin (PCF)-blended sponge sheets and wound healing effects of these sheets in rats were investigated. We excised the skin of a rat, including the dermis, approximately 2×2 cm in size. The wound was covered with PCF-blended spongy sheets. The spongy sheets absorbed the exudate, and gained flexibility and softness. Histopathological inspection of the wound 12 days later showed the increase of a vascular ingrowth and the absence of inflammatory cells. Regeneration of the skin around the wound was faster than that of the control. We also tested wound healing effects of PVA, Chitosan and Fibroin, alone or in various combinations. Wound healing was accelerated in the order of PVA/Chitosan/Fibroin (PCF)-blended sponge > Chitosan/Fibroin (CF)-blended sponge ≥ Fibroin (F) sponge > PVA/Chitosan-blended sponge (PC) > Chitosan (C) sponge.

Key words : Wound healing effects, PVA/Chitosan/Fibroin (PCF)-blended sponge, Histopathological inspection, Silk fibroin

Introduction

The natural materials such as chitosan and silk fibroin have been proved to be invaluable in the field of biomedical engineering and biotechnology, with a wide variety of applications ranging from skin and vascular grafts to substrates for mammalian cell culture (Chen et al., 1994; Koyano et al., 1998). For example, silk fibroin from the Bombyx mori is structure proteins possessing unique physical properties and their applications have recently

been considered for the generation of biomedical products such as an enzyme-immobilization materials and blend materials (Demura et al., 1991; Hanawa et al., 1995a, 1995b; Kang et al., 2000). In addition, fibroin can easily be obtained in an aqueous solution, and transformed into gel, powder and film, etc. It is desirable that fibroin aqueous solutions are obtained only by dissociating intermolecular bonding without breaking the peptide chains because the peptide chain is required for the production of surgical structure. Regenerated silk fibroin has been also proved to have blood compatibility (Minoura et al., 1995). Chitin is one of the most abundant polysaccharides, with a widespread distribution in invertebrates and lower plants (Okamoto et al., 1995). Deacetylation of chitin by alkaline hydrolysis yields chitosan, which is one of a few natural cationic polyelectrolytes. From the solubilized chitin and chitosan, membrane, fiber and gel can be made. As chitin can be digested by lysozyme in living tissues, absorbable surgical suture has recently been developed from it. As chitin contributes to the healing process of the wound, a wound dressing was also developed (Rao and Shama, 1997; Peter, 1995).

On the other hand, poly vinyl alcohol (PVA) is a nontoxic water soluble synthetic polymer. It processes many useful characteristics including excellence in film formation, high hydrophilicity, impact strength, crystalline modulus, and alkaline resistance. PVA has been widely utilized in chemical and medical industries for the productions of fibers, films, sponges, coatings, cosmetics, pharmaceuticals, etc. Moreover, PVA hydrogels have been investigated as alternatives to biological tissues, and used as an immobilizing matrix against microorganisms and enzymes (Liu et al., 1997; Cho et al., 1996; Kageyama et al., 1994). Blending with PVA has a great affinity for skin and extracellular matrix materials. We expected that blending PVA/Chitosan/Fibroin (PCF) might produce a highly elastic hydrogel type spongy membrane facilitating collagen synthesis of skin tissue.

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In this study, we attempted to modify silk fibroin physically for its potential application in biomedical field by blending it with the matrix mentioned above such as PVA. Chitosan and fibroin have been further studied to obtain new materials and to use them as covering sheets for a wound healing. The present work reports an *in vivo* evaluation of wound healing effects of PCF.

Materials and Methods

Materials

Dried cocoons of *Bombyx mori* reared on the farm affiliated with Rural Development Administration in Korea, were used as the raw materials. The raw materials were degummed twice with 0.5% w/w marcellus soap and 0.3% (w/w) $NaHCO_3$ solution at 100°C for 60 min. and then rinsed with deionized hot water. We will call that grade as fibroin. The loss of raw cocoons in the process of boiling was 24%. Chitosan powder was purchased from Aldrich chem. Co., Ltd (Louis, USA). It has a N-acetylation degree of 79% according to elemental analysis. PVA powder was obtained from Yakuri pure chem. Co., Ltd (Tokyo, Japan). It has a degree of polarization of 1500 and a saponification degree of 99 mol%. Both powders were used without further purification.

PCF preparations

Regenerated silk fibroin (F) solution: Degummed silk was dissolved in the following mixed solution. Anhydrous calcium chloride (266.4 g) was weighed precisely and dissolved 346 ml of pure water and an aqueous solution of calcium chloride and water (molar ratio of 1:8) was prepared. Then, 280 ml of ethanol was added to the aqueous solution above, and an aqueous calcium chloride-water-ethanol solution with a molar ratio of 1:8:2 was prepared. Degummed silk (Fibroin) was added to 35 g of aqueous calcium chloride-water-ethanol at 95°C for 5 hr. After dialysis of distilled water for 4 days, the solution was filtered and the regenerated aqueous silk fibroin (F) solution was obtained. The concentration of the solution was approximately 2% (w/w), and concentrated to 4% by mixing air stream from an electric fan. This solution was referred to as S_1.

Chitosan/Fibroin (CF) aqueous solution: 4 g of chitosan powder was dissolved in 100 ml of 0.75% (w/w) acetic acid (Guaranteed reagent, Junsei chem. Co., Ltd) at 60°C, and the filtrate of this solution was obtained (C). One hundred ml of this chitosan solution was added to the same concentration of fibroin aqueous solution at 60°C and adjusted at pH 5.0~5.5. with 0.1 N sodium hydroxide solution using pH meter.

PVA/Chitosan (PC) aqueous solution: This solution was

prepared in the same method mentioned above. That is to say, the same concentration of the 100 ml of PVA in pure deionized water at 60°C was dissolved and adjusted at pH 5.0~5.5 using pH meter. This blended solution was referred to as S_2.

Preparation of PVA/Chitosan/Fibroin (PCF) spongy sheet: The PVA/Chitosan/Fibroin (PCF) spongy sheet was prepared by lyophilization of this blend aqueous solutions. First, 35 ml of the S_2 sample mentioned above was added to 15 ml of S_1 sample and poured together into a polystyrene dish (diameter 14 cm), then carefully mixed, placed on a homebuilt balance control plate and left in the freezing control box at -70°C to make a homogeneous blending membrane. The porous spongy sheet with a honeycomb-layer like structure was punched with 20 μm hole. The spongy sheet was irradiated with a 15 W ultraviolet lamp for 1 hr at a distance of 20 cm to make it insoluble in water, and to induce intermolecular cross-linkage (Kim *et al.*, 1996). Then, each spongy sheet was placed in a polystyrene dish, and sterilized with ethyleneoxide gas. The other spongy sheet as prepared by the same techniques as mentioned above.

In vivo animal tests

Adult male Sprague-Dawley rats, purchased from the animal center of the Sam-Yuk in Korea, weighing 350 ± 5 g, were used in these experiments. Each rat was caged alone and allowed chow and water ad libitum. Surgery and dressing changes were performed with the animals anesthetized with 1 g/kg intraperitoneal urethane (Sigma) in 0.4 ml normal saline. The skin over the dorsal area was shaved completely and application fields were outlined with marking pen just prior to skin incisions. There were five groups each consisting of 5 animals. The skin wound of full thickness was prepared by excision (2×2 cm) of the dorsum of rat. Then, excised wound was covered with different spongy-typed dressing materials (C, F, PC, CF and PCF). As a control, conventional gauze dressing was applied on the same experimental conditions. The rats of each group were daily scrutinized for 12 days after application for the observation of healing process.

Histologic evaluations

After 12 days, skin samples from the rats were taken. The central portion of underlying tissue was taken and fixed in 10% buffered formalin. Each specimen was embedded in a paraffin block and thin sections were prepared, stained by the hematoxylin-eosin and masson's trichrome method (Peter, 1995). The wound healing effect was examined histologically under a light microscope. Also, the % area of collagen contents on the areas stained with trichrome of the wounds as determined using the Image Analyzer (Zessis IBAS 2.5, Germany) attached to the computer

with CILES software.

Electron microscopy

An arbitrary 50 mm-long segment of each samples as mounted across the 30 mm gap of a U-shaped aluminium sample holder and wrapped with double sided adhesive tape. The test samples as coated with gold-palladium about 200 Å thick. This proved to be a suitable coating, in both thickness and type of coating material, for revealing the details of the surface of the material when stretched inside the SEM chamber. The electron microscopy for PVA/Chitosan/Fibroin (PCF)-blended spongy sheets was performed on scanning electron microscope (Hitachi, Nissei Sangyo America, Mountain View, Calf) operated at 75-kV accelerating voltage.

Statistical analysis

All data are expressed as the mean±S. D. The evaluation of statistical significance was determined by the student's t-test and one-way ANOVA test using a standard package for microcomputers. Statistical significance was considered at the level of $p<0.05$.

Results and Discussion

Wound healing is a complex phenomenon involving induction of an acute inflammatory process by wounding, regeneration of parenchymal cells, remodeling of connective tissue and collagenization (Cohn et al., 1992). Connective tissue matrix, particularly collagen, remodeling of the acquisition of wound strength are the ultimate events of orderly wound repair. In the current work, we focused on the effects of functional polymers such as fibroin, chitosan and PVA in wound healing processes with a particular interest in the aspects of wound collagenization.

First, toxicity of the dressing materials was tested. Rats covered with different dressing materials used in this study did not show any toxic effect when they were used to cover wound in rats. All rats gained weight during the period of observation, and the average weight of control and treated groups did not show significant difference until the end of the study (data not shown). The inflammatory reactions of the skin in the groups treated with F, C, CF, PC and PCF tended to be less marked than those of the control group throughout the inspection periods. Histological examination of the wound was carried out at 12 days after treatment (Fig. 1). As illustrated in Fig. 1A, wound site of control group was not fully epithelialized, and the neodermis was not reconstructed. Unevenness of epidermis near the ulcer was observed in the untreated control group, whereas the PCF sheets-treated groups did not show such phenomena. But, PCF sheets-treated group
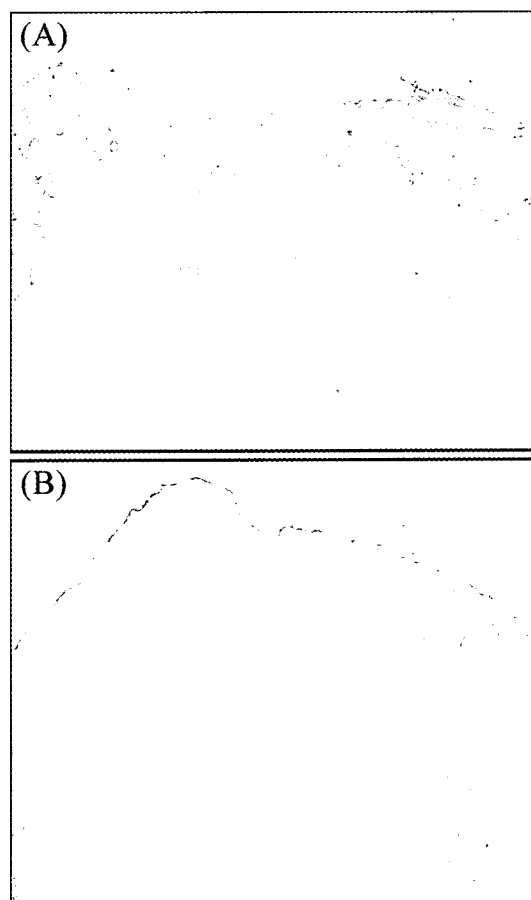

Fig. 1. Histologic appearance in the surface of dermis 12 days after placement. The skin over the dorsal area was shaved completely and application fields were outlined with marking pen just prior to skin incisions. The full thickness skin wound was prepared by excision (2×2) of the dorsum of SD rat. A, Wound site of control group was not fully epithelialized, and did not reconstruct the neodermis. Unevenness of epidermis near the ulcer was observed in the untreated control group. B, PCF-treated site. By this time the wound had almost completely closed by epithelialization and contraction. Hematoxylin-eosin stain were original magnification ×40.

showed fibroplasia, followed by tissue remodeling and scarring (Fig. 1B). After these initial events, a synthesis of collagen was increased in the PCF sheet-treated group and a repair system was initiated. As a result, the surface of epidermis became even. Later 10 days, there were significant differences between the treated and control group. For example, while little evidence of vascular ingrowth into the uncovered wound section was noted, histological examinations of F sheets-covered wounds revealed minimal or no inflammatory reaction at any point. Similar histologic appearances were seen in the CF-covered specimens. Particularly, the PCF sheet-treated group showed the greatest wound healing among all the treated groups.

The histologic findings in the rats treated with PCF

sheets imply that PCF sheet is effective in wound healing. Considering these histologic findings and previous reports on biological activities of chitosan and fibroin (Chvapil, 1982), wound healing effects of these materials can be improved by concurrent increase in the production of collagen. During the treatment, continued accumulation of collagen and proliferation of fibroblast were observed (Fig. 2). Examination of subepidermal tissue revealed numerous collagen bundles arranged parallel to the dermal-epidermal junction. This configuration implies the organized reconstitution of dermis without excessive scar formation.

Masson's trichrome stain of healed scar, which stains collagen blue, shows the dense collagen with only scattered vascular channels. Collagen is the most common protein in animals, which provides the extracellular framework for all multicellular organisms. Fibroblast and collagen, ultimately provides the tensile strength of heal-
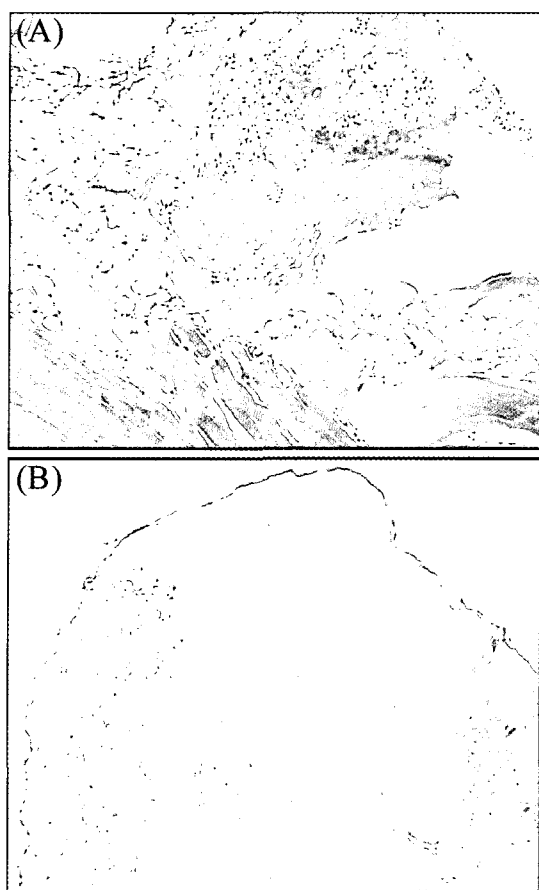

Fig. 2. Typical histologic appearance of collagen production 12 days after placement. A, Control (non-treated). Dense collection of inflammatory cells and vacuole represent in tissue. No evidence of vascular ingrowth is seen. B, PCF-treated site. Inflammatory cells are absent and vascular ingrowth has increased. Masson's trichrome stain (arrows); original magnification ×40.

ing wounds (Kuroyanagi *et al.*, 1987; Hino and Okamura, 1985). The epidermis recovered its normal thickness, and differentiation of surface cells yielded a mature epidermal architecture with surface keratinization. The contents of collagen produced in the wound area is shown in Table 1. The mean percentage of collagen production was $47.49 \pm 5.77\%$ and $65.22 \pm 4.23\%$ in CF and PCF-covered rats, respectively. These values were higher than the relative content in the control group, which was $2.42 \pm 0.32\%$. Regeneration of the skin around the wound was faster in PCF sheets treated group than those in the control, showing that wound healing was accelerated in the order of PCF-blended sponge > CF-blended sponge > F-sponge > PC-blended sponge > C-sponge. However, whereby PVA promotes wound healing processes, PVA may transform physical properties of PCF sheet that is unknown. It may possible be due to transformation of physical property such as flexibility. After the freeze-drying of the PCF solution, smooth, soft, and flexible planar sponges were obtained. The scanning electron microscopical examinations of a PCF-freezed sponge were performed (Fig. 3). The surface of CF-blended sponges was very crude and rough. The separation of phase layer between upper and lower layer was observed under SEM (Fig. 3A). On the other hand, the surface of PCF-blended sponges was uniform. The thickness of the spongy layer varied from 100 to 200 μm and its pore sizes ranged from 10 to 30 μm (Fig. 3B). The spongy sheet was punched mechanically to make many holes for a honeycomb-layer like structure. The PVA aqueous solution, which was applied to spongy sheet with the honeycomb-layer like structure both in the holes and the unique layer. It suggested that water permeability might be increased by adding PVA in CF solutions. Additions of PVA to CF increased flexibility and softness. The CF and Fibroin/PVA blended polymers are already well studied (Qian *et al.*, 1996; Chen *et al.*, 1997). They could form a hydrogen bonding within a novel nat-

Table 1. Quantitation of collagen contents produced in wound area 12 days after dermal graft by using image analysis

Dermal graft composition	Collagen content (%)
Control (non-treated)	2.42 ± 0.32
Chitosan (C)	23.87 ± 7.04^a
Fibroin (F)	46.05 ± 4.53^b
PVA+Chitosan (PC)	$30.54 \pm 3.25P^a$
Chitosan+Fibroin (CF)	47.49 ± 5.77^b
PVA+Chitosan+Fibroin (PCF)	65.22 ± 4.23^c

Values are the mean $\pm$ S. D. (n=5). [a] Significantly different from the non-treated group (p < 0.05). [b] Significantly different from the non-treated group (p < 0.01). [c] Significantly different from the non-treated group at (p< 0.001).
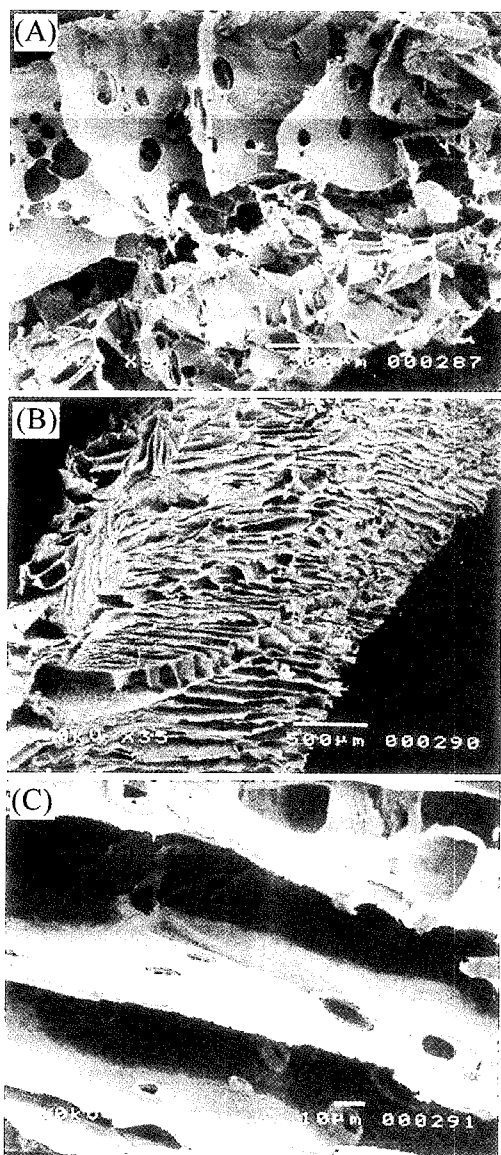
Fig. 3. Surface of lyophilized sponges using scanning electron microscopical examination. A, Electron micrograph of CF-blended sponges. Scale bar is 500 μm, magnification ×35. B, Electron micrograph of PCF-blended sponges. Arrow indicates a holes to make a honeycomb-layer like structure. Scale bar is 500 μm, magnification ×35. C, Enlargement of electron micrograph of PCF-blended sponges. Scale bar indicates 10 μm, magnification ×750.

ural semi-interpenetrating polymer and network (Sugimoto *et al.*, 1998). The amino group of silk fibroin mainly forms semi-interpenetrating network through hydrogen bonding within the samples by blending these samples. Moreover, among the three elements consisting the blended membranes, silk fibroin component is considered to be the most important. All these analyses suggest that mixing of these component may accelerate wound healing by rendering intermolecular interaction strong. Therefore

PCF has the suitable characteristics as wound covering materials. The shape and formulation of spongy sheet appear to be important for its wound healing effects. It should be emphasized that PCF promotes wound healing processes by facilitating collagenization. As noted, the deposition of connective tissue matrix, particularly collagen, is an important step toward orderly process of wound repair.

These PCF membranes could be utilized in clinical dermatology. For its clinical uses, effective doses of PCF sheets should be determined. The doses in this study were effective in treating wounded rats, but the optimal doses and duration times for the clinical uses need to be further determined.

Acknowledgments

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