Studies on gelatin-based sponges. Part III: A comparative study of cross-linked gelatin/alginate, gelatin/hyaluronate and chitosan/hyaluronate sponges and their application as a wound dressing in full-thickness skin defect of rat

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Novel cross-linked sponges composed of gelatin/alginate and gelatin/hyaluronate and chitosan/hyaluronate (GH, GA and CH, respectively) were prepared and compared. Six different sponges with or without silver sulfadiazine (AgSD) were applied on the full-thickness dorsal skin defect of Wistar rat. The histology and epidermal wound healing rates of the skin defects were investigated by light microscopy and computerized morphometry 5 and 12 days post-operatively. In our full-thickness wound model (diameter 1 cm), the AgSD-impregnated sponges showed good wound healing performances on the whole. However, there appeared meaningful differences of wound healing between the gelatin-based sponges (GH, GA) and the CH. GH with AgSD was found to show the best wound healing properties as a wound dressing resulting from histological findings and computerized morphometric analysis of epidermal healing.

1. Introduction

Even though there have been a number of reports on the development of new materials as wound dressings, it would be regrettable only to find out that no single material is ideal [1–3]. For an ideal wound dressing, materials should have flexibility, durability, adherence, a capability of absorbing wound exudate and protecting the lesion from dehydration. From an engineering viewpoint, it should also be easy to handle and to apply, comfortable when in place and cost-effective. More than anything else, wound dressing materials should be non-toxic, non-antigenic, and stable (insoluble, non-resorbable) during a few days of contact with the wound.

There have also been many comparative studies on synthetic wound dressings such as polyurethane (PU), poly(vinyl alcohol) (PVA), poly(hydroxyethylmethacrylate) (pHEMA) other than biological wound dressings, for example, pigskin, bovine collagen and chitin etc. [4–8]. We have already reported on the wound healing effect of modified chitin sponge and simple cross-linking method of gelatin-based sponges [9–12]. Among many biomaterials suitable for an ideal wound dressing, we selected gelatin, sodium alginate, chitosan and sodium hyaluronate to prepare sponges composed of each component, then evaluated each as a wound dressing in the rat model.

Recently, gelatin is known to exhibit activation of macrophage and a high haemostatic effect. Gelatin is practically more convenient than commercially used collagen because a concentrated collagen solution is extremely difficult to prepare from native collagen and furthermore gelatin is far more economical than the collagen [13–17]. Alginate is so hydrophilic, biocompatible and relatively economical that it has often been used in medical applications such as wound dressings, scaffolds for hepatocyte culture and surgical or dental impression materials. Alginates are also known to break down to simpler glucose type residues and be totally absorbed [18–20]. Hyaluronic acid (HA) consists of 2-acetamide-2-deoxy-α-D-glucose and β-D-glucuronic acid residues linked by alternate (1–3) and (1–4) glycoside bonding. HA, the component of the extracellular matrix, has a high capacity for lubrication, water sorption and water retention and influences several cellular functions such as migration, adhesion and proliferation. Recent biomedical applications of HA include ophthalmic surgery, arthritis treatment, wound healing, coating and as components of implant materials

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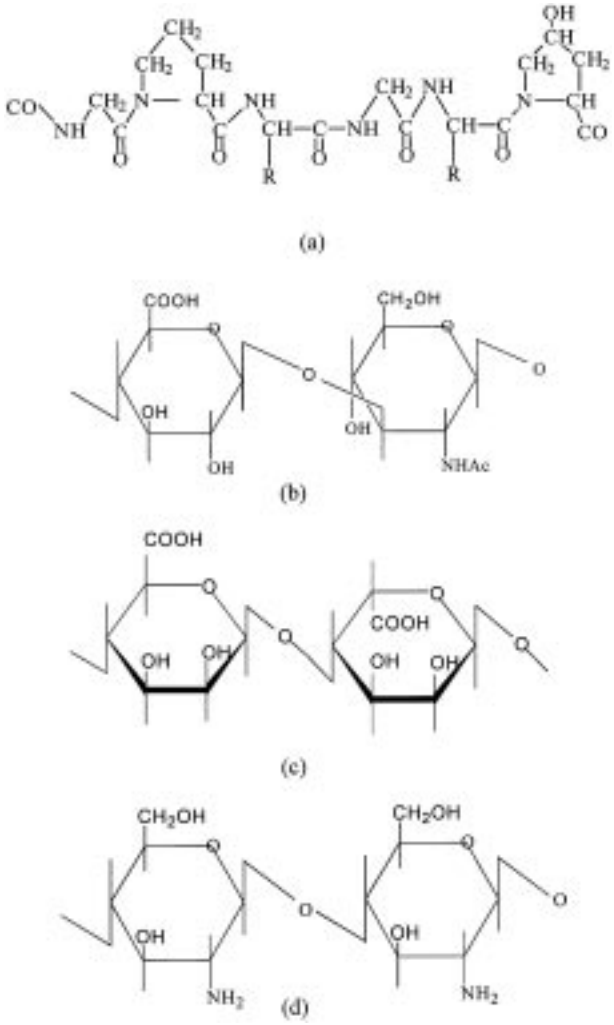

Figure 1 Representative structure of selected compounds: (a) gelatin, (b) sodium alginate, (c) chitosan, (d) hyaluronic acid.

[21, 22]. Chitosan, a deacetylated form of chitin, has a subunit of (1, 4)-linked 2-amino-2-deoxy-β-D-glucan. The viable bioactivity of chitosan has made it an interesting biomaterial. Chitosan has been applied in hematology, immunology, wound healing, drug carrying and cosmetics [23, 24]. The molecular structures of these compounds are shown in Fig. 1.

There have been few reports on the applications of gelatin-based sponges to wound dressing as well as comparative studies of these sponges containing hyaluronic acid until now. We have already reported on the novel preparation method of gelatin-based sponges containing alginate or hyaluronate and chitosan-based sponges including hyaluronate [10, 11]. The objective of the present study is to compare three newly-prepared sponges as wound dressings in the rat skin defect model in terms of epidermal healing rate. The ultimate goal of this study is to find out the optimum sponge among the three for an application as a possible wound dressing.

2. Materials and methods

2.1. Materials

Gelatin was purchased from Kyung-gi Gelatin Co. (Seoul, Korea). It was alkaline-processed gelatin with an isoelectric point of pH 4.96 and theoretical molecular weight of 100 000. Sodium alginate was purchased from Aldrich Co. (Milwaukee, WI). 1-ethyl-(3-3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Sigma Co. (St. Louis, MO). Silver sulfadiazine (AgSD) was donated by Dong-Wha Pharmaceutical Co. (Seoul, Korea). Chitosan ($M_v = 500\,000$, degree of deacetylation $= 76\%$) was purchased from Tokyo Kasei Co. (Tokyo, Japan). Hyaluronic acid, sodium form ($M_v = 1\,700\,000$) was obtained from Pacific Chemical Co., Ltd (Seoul, Korea). Water was distilled and deionized with the Milli-Q System (Millipore, MA, USA). Other chemicals were reagent grade and used without any further purification.

2.2. Preparation of cross-linked gelatin/alginate (GA) or gelatin/hyaluronate (GH) sponge

Cross-linked gelatin/alginate or gelatin/hyaluronate sponge was made in two steps as previously reported [10, 11]. Briefly, 1 wt % (w/w) aqueous solution of gelatin and sodium alginate, respectively, was dissolved in double distilled water at 50 °C for 3 h. Each solution with certain mixing ratios of gelatin and sodium alginate was stirred for 30 min at room temperature, frozen to − 70 °C overnight and lyophilized at − 50 °C for 24 h. This soluble sponge was cross-linked with EDC by immersing the soluble sponge in 90% (w/v) aqueous acetone containing 0.1 ∼ 0.5 wt % (w/v) EDC for 24 h at room temperature, while shaking slowly.

2.3. Preparation of cross-linked chitosan/hyaluronate (CH) sponge

Mixtures of HA and chitosan were prepared by dissolving two components in various ratios in 15% formic acid aqueous solution. The concentration of chitosan and HA in the solution was 1 wt %. This solution was filtered using a glass filter. When water was added to the solution containing HA and chitosan, the pH increased so much that precipitates were formed at pH 2 ∼ 3. Then the precipitates were centrifuged for 30 min at 12 000 rpm. The obtained product was poured into a petri dish and frozen (− 76 °C) for 6 h. Then, the petri dish was dipped into water to remove any remaining formic acid that did not participate in the formation of polyelectrolyte complex. After washing with water, the petri dish containing the precipitates was kept at − 76 °C for one day and then freeze-dried to prepare porous cross-linked sponges

2.4. Application of sponges on rat skin defect wound

The dorsal hair of the Wistar rat (180 ∼ 200 g) was shaved and anaesthetized with Entobal®. After disinfection of the skin with Betadine®, a full thickness skin wound of 1 cm in diameter was prepared by excising the dorsum of a Wistar rat. The excised wound was covered with ethylene oxide (EO) gas-treated gelatin/alginate or gelatin/hyaluronte sponge (1 cm × 1 cm) with or without AgSD (0.4 mg/cm^2). Then, a sterilized elastic band was employed to fix the sponges. As a control, Vaseline gauze was applied on a skin wound. At the 5th and 12th

postoperative day, the Wistar rats were sacrificed for gross and histological findings.

2.5. Morphology of sponge

The morphologies of three sponges were investigated by scanning electron microscopy (SEM, JEOL–6400F). Specimens were placed on a Cu mount and coated with gold-coating apparatus. The sponge microstructures were investigated by geometrical measurement on SEM. The porosity, average diameter of pores and wall thickness (i.e. the average distance between neighboring pores) were measured using an Image Analyzer (Bum Mi Universe Co., Ltd, Seoul, Korea).

2.6. Histological analysis of wound

A fixation in 10 wt (v/v) % neutral-buffered formalin was immediately carried out after macroscopic observation of skin wound covered with sponges. A skin wound tissue was biopsied in a strip of about $0.5\,\text{cm} \times 2.5\,\text{cm}$, embedded in paraffin wax, sectioned in $4\,\mu\text{m}$ size, followed by staining with hematoxylin-eosin. The resulting healing effects were histologically investigated.

2.7. Computerized morphometric analysis of epidermal wound healing

Each microscopic wound section was placed on the stage of the microscope, where the image was then displayed on a video screen via CCD camera (Mitsubishi, Japan) interfaced to a Compaq-Pentium computer. The percentage of wound resurfacing was determined by measuring on-screen the distance from the right wound margin to the left wound margin using an image analyzing program (Bmi Plus 1.14). Then, the length of newly regenerated epithelium across the surface of the wound was determined. This length was defined as the sum of the new epidermis growing out from the left and right margin of the wound [25].

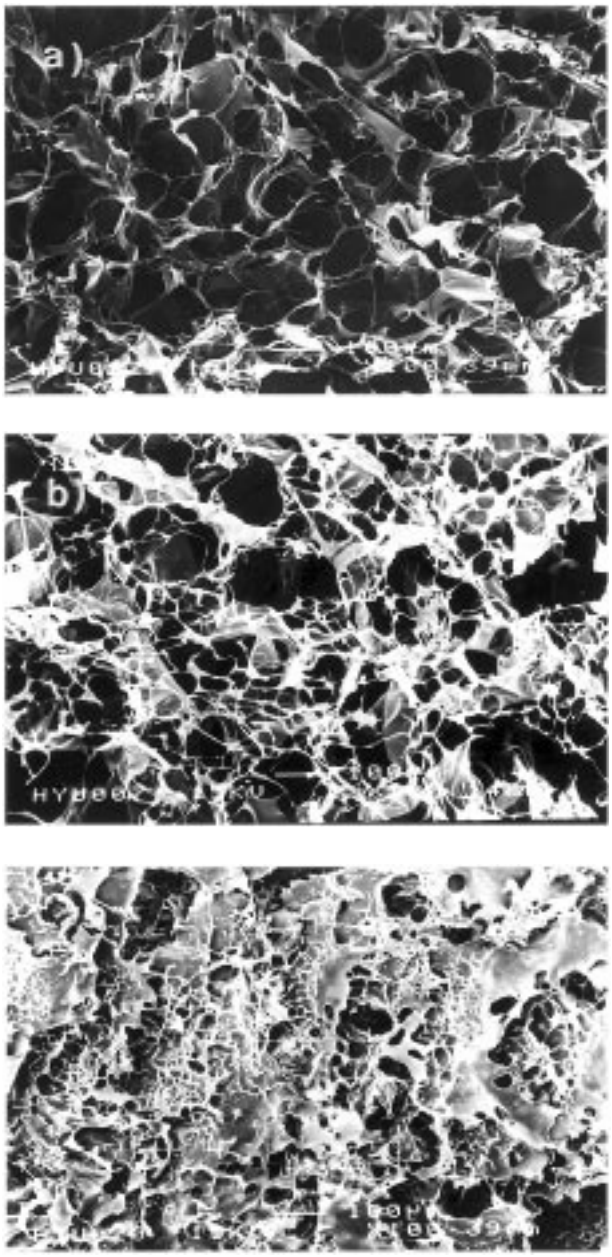

Figure 2 Surface SEM of (a) GH, (b) GA and (c) CH (original magnification × 100).

3. Results and discussion

3.1. Morphological and physical characteristics of the sponges

The surface morphology of individual sponges was markedly different, as shown in SEM images (Fig. 2). While GH and GA displayed two different network structures within a sponge, CH showed a uniform network structure with a narrow pore size distribution. Gelatin-based sponges show the inter-connected network structure of gelatins characterized by the membrane-like structure while alginate was noticed by a fibrous structure. In the case of CH which were composed of high molecular weight polymer chitosan and hyaluronate, however, the sponge showed more compact network than the others. In addition, the cross-sectional SEM images are shown in Fig. 3.

Some physical and morphological characteristics of the tested sponges are given in Table I. The porosity of sponges ranged between $40 \sim 70\%$ with an average pore size of $20 \sim 160\,\mu\text{m}$. The morphologies of these sponges seemed to be highly dependent on the mixing ratios of each solution in addition to the quenching temperature, $-76\,^{\circ}\text{C}$, where quenching effect was demonstrated well elsewhere [23, 24].

As far as the water uptake abilities of the sponges were concerned, GH sponge seemed to be the best among the tested sponges, followed by GA and CH as shown in Table I. The water uptake abilities of the sponges increased with the content of hyaluronate or alginate which were so hydrophilic as to make the sponges retain a high content of water. CH sponges showed the poorest water uptake, compared with the other two sponges. On touch, GH sponge was the softest, followed by GA and then CH.

3.2. Histological studies of wound healing

Figs 4 and 5 exhibit the histological results of the tested sponges. Histological cross-sections of sponges with or

Figure 3 Cross-sectional SEM of (a) GH, (b) GA and (c) CH (original magnification × 40).

without AgSD and Vaseline gauze as a control group were studied after 5 and 12 days of covering.

3.2.1. Case without AgSD in sponge

As shown in Fig. 4a and c, the GH and GA without AgSD after 5 days, the surface is covered by a thick zone of necrotic tissue admixed with acute inflammatory exudate. The exuberant granulation tissue is noted under the thick layer of the exudate. In the case of the CH without AgSD (Fig. 4e), it shows thicker layers of fibrinous materials alternated with cellular neutrophilic infiltrates over the skin defect. The Vaseline gauze showed a very poor status of healing; a congested blood fibrinous exudate rich in neutrophils and macrophages and an irregular rough dermal surface (Fig. 4g). In this short-term histological comparison, we could reach an agreement that the GA, GH, CH and Vaseline gauze, in this order, showed wound healing effect.

At the 12th post-operative day, macroscopically, the wounds seemed almost re-epithelialized. Histological studies, however, tell us that each case of sponges without AgSD are somewhat different in healing stage. The gelatin-based sponges, GH and GA, showed similar histological characteristics in that the skin defect was almost covered by thin epithelium whose epidermal cells were is as incipient stage of proliferation and gradual differentiation, and moreover the dermis showed a healing fibrovascular tissue with fibroplasia, collagen laydown, and dilated blood vessels. In spite of these qualitative similarities, we could find that there were conspicuous differences of wound healing rates by measuring the length of the newly regenerated epithelium growing out from both margins with computerized morphometric analysis, the results of which are displayed in Fig. 5. Even without AgSD, GH showed the highest healing performance among the tested sponges. In this regard, the overall score of healing competency of the four specimens could be valued in the order of GH, GA, CH and Vaseline gauze.

3.2.2. Case with AgSD in sponge

As shown in Fig. 6a, c, and e at 5th day of post-operation, we could observe that the amount of acute inflammatory exudates above the dermis became lessened compared with the counterparts mentioned previously in Section 3.2.1, the reason being considered to be the antibacterial effect of incorporated AgSD. After the 12th day, enhanced wound healing effects were observed in three sponges compared with sponges without AgSD, as shown in Fig. 4b, d and f.

In the case of the gelatin-based sponges, the wound was almost completely covered with a thin layer of epidermis whose basal cell began to proliferate into dermis, showing characteristic rete ridges and with

TABLE I Physical and morphological characteristics of tested sponges

Sample Code§	Porosity* (%)	Average pore size (μm)†	Wall thickness (μm)	Water uptake‡ (g H_2O/g sponge)
GH	66.8 ± 0.3	152 ± 5.7	2 ± 0.4	40 ± 2.2
GA	53.4 ± 0.5	94 ± 1.2	5 ± 0.9	35 ± 3.2
CH	42.5 ± 0.2	25 ± 3.4	9 ± 0.8	10 ± 4.4

*Porosity was obtained from areal analysis between the pore zone and matrix zone by the Image Analyzer Program.
†Average pore size was also calculated by measuring the size of 60 pores with the Image Analyzer tools.
‡Water uptake ability was calculated from the weight difference between the wet and dry state of the sponges.
§G: gelatin, H: hyaluronate, A: alginate, C: chitosan.

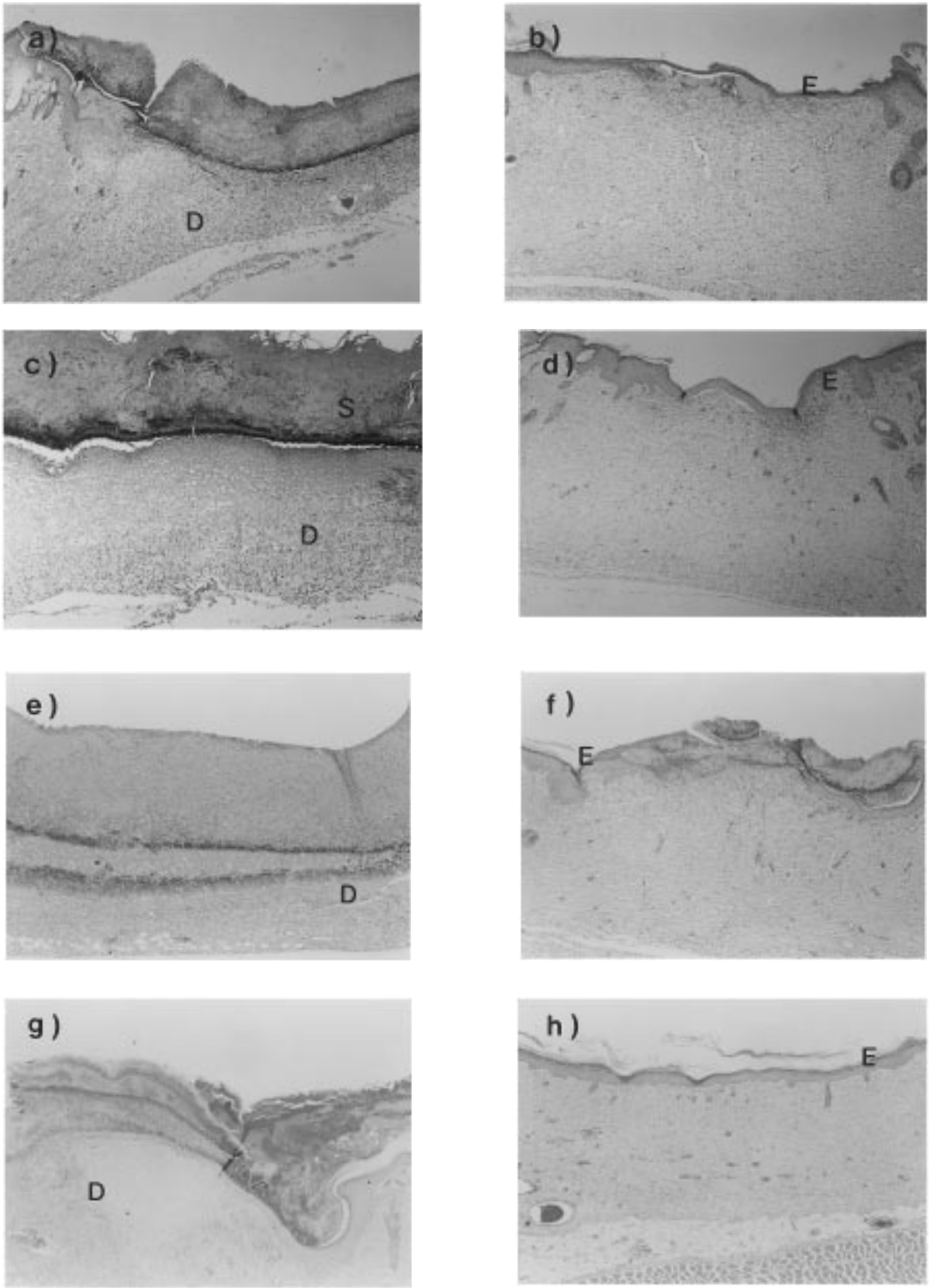

Figure 4 Histological appearances of a wound surface on the rat's dorsum covered with 3 types of sponges without AgSD after 5 and 12 days of operation. Hematoxylin and eosin (H&E) stain. × 40. (a–b) GH, (c–d) GA, (e–f) CH and (g–h) Vaseline gauze (E: epidermis, D: dermis, S: sponge).

prolific granulation tissue in dermis. However, CH still showed thin atrophic epidermis, focal hyperkeratosis and neutrophilic infiltrates. These histological differences were also confirmed by measuring the regenerated epidermis, morphometrically, results are shown in Fig. 7.

In fact, the gelatin-based sponges including GH and GA showed similar histological characteristics, whereas CH showed poor performances of wound healing even with AgSD. Compared with vaseline gauze as a control group, the healing process of gelatin-based sponges could be evaluated to be fast and stable. The reasons for this are assumed to be that the higher water uptake ability of GH and GA may incur rapid absorption of hemorrhagic exudate at the wound bed, compared with

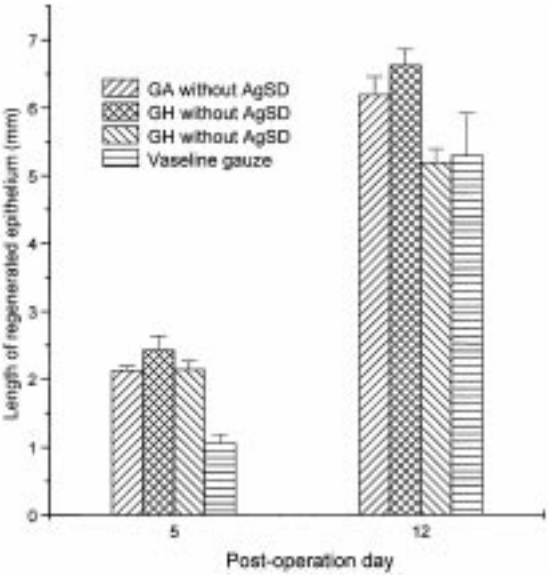

Figure 5 Morphometric analysis of epidermal wound healing by GA, GH, CH in which there is no AgSD, and Vaseline gauze as a control in full thickness wound with a diameter of 1 cm at the 5th and 12th post-operative day ($n = 3$).

relatively lower CH and vaseline gauze. In other words, GH and GA with highly porous structure were thought to allow the overflowing hemorrhagic exudate at the instant of wound formation to be immobilized within the sponge structure, thus preventing the formation of mobile body fluid pockets.

Another reason for the enhanced wound healing effect of the tested gelatin-based sponges could be considered that after absorbing the hemorrhagic exudate, the AgSD-containing sponges might slowly release the drug into the wound, based on our previous results on the drug release behavior of AgSD-containing sponges which showed sustained release behavior of AgSD for up to 4 days [10, 11].

A final point that we have to make in our protocols of animal experiment was that the wound healing effect seemed to depend to a large extent on the use of antibiotic rather than the properties of the biomaterials used when preparing the sponges. Although we could confirm the effect of the material itself in our small diameter full-thickness rat model, we found the effect of alginate and hyaluronate on the wound healing, using

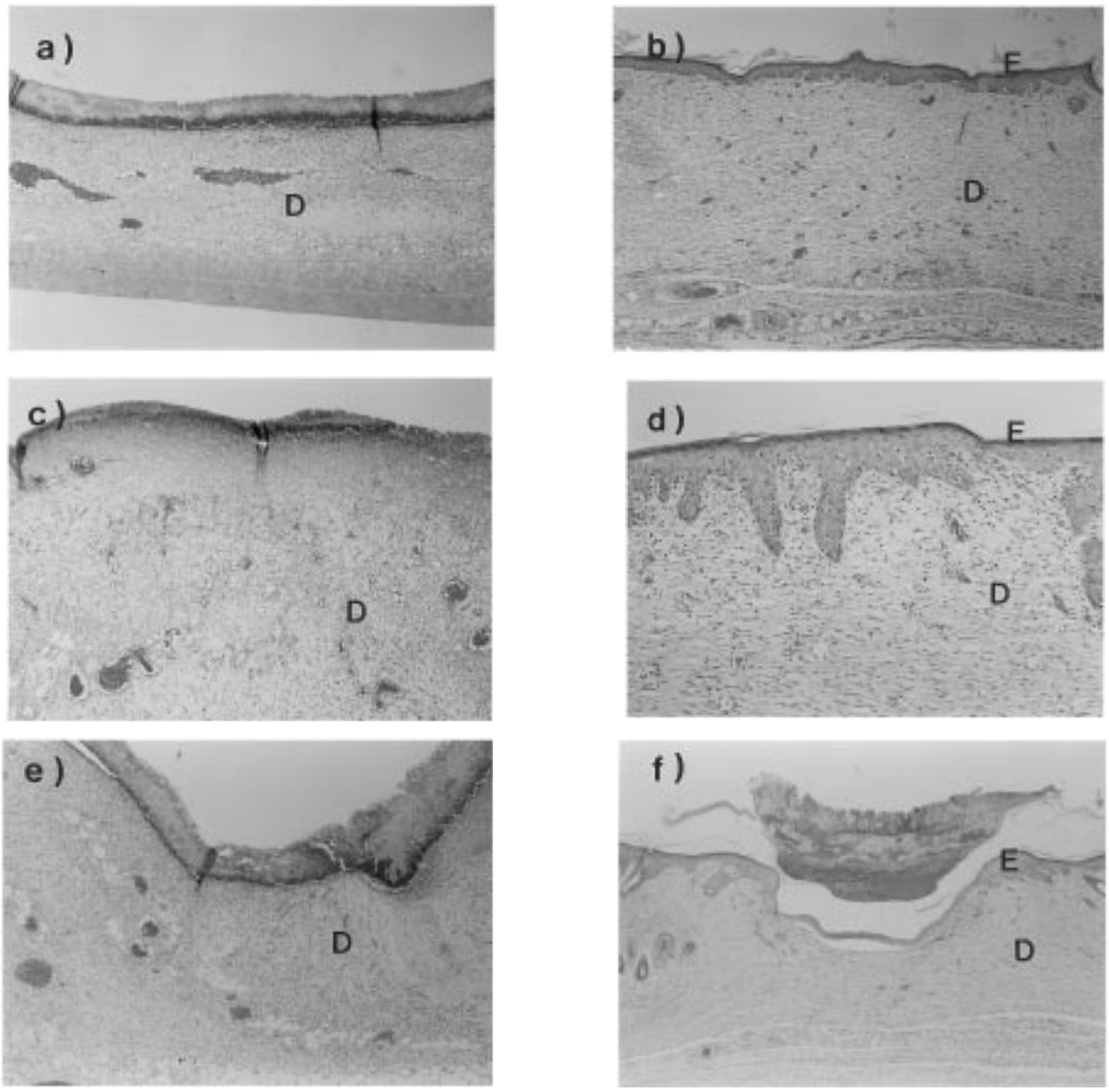

Figure 6 Histological appearances of a wound surface on the rat's dorsum covered with 3 types of sponges with AgSD 5 and 12 days post-operation. Hematoxylin and eosin (H&E) stain. Original magnification × 40. (a–b) GH, (c–d) GA, (e–f) CH. (E, epidermis, D, dermis, S, sponge).

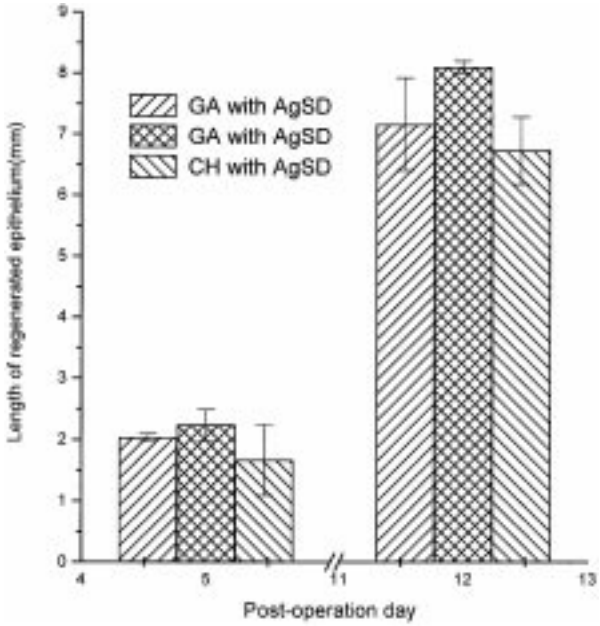

Figure 7 Morphometric analysis of epidermal wound healing by GA, GH, CH in which there is AgSD in full thickness wound with a diameter of 1 cm at 5th and 12th post-operative day ($n = 3$).

larger diameter full-thickness skin defect model, which will be reported soon.

4. Conclusions

This work showed that using biodegradable and biocompatible natural polymers, gelatin, alginate, hyaluronate and chitosan, novel cross-linked sponges were successfully prepared for application as a wound dressing. These sponges with porosity of 50 $\sim$ 70% and average pore diameter of 90 $\sim$ 160 μm showed a mixed network of each added component, except CH which showed more compact and narrow pore size distribution. The increased porosity of the gelatin-based sponges was attributed to the addition of alginate or hyaluronate, resulting in an increased water uptake ability that ranged 10 $\sim$ 40 times of the weight of original sponge.

Results on the application of these natural polymeric sponges to wound dressing materials in rat's skin defect model indicated that the AgSD-impregnated gelatin-based sponges containing sodium alginate or sodium hyaluronate made the skin defect almost re-epitheliaze at the 12th post-operative day with a minor infection and good integrity of dermis. In our experimental model, GH with AgSD was found to show the best wound healing properties as a wound dressing from all the histological findings and computerized morphometric analysis of epidermal healing.

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