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알지네이트 스폰지 및 하이드로젤의 습윤성 창상치유 특성

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Moisture Wound Healing Characteristics of Alginate Sponge and Hydrogel

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초록: 건조/가교법으로 제조한 알지네이트 스폰지 및 하이드로젤의 물리적, 생물학적 특성 및 창상치유 특성을 고찰하였다. 하이드로젤은 스폰지 대비 높은 평형 함수율을 보유하였고, 자체적으로 수분을 함유하고 있기 때문에 상대적으로 우수한 습윤 창상치유 환경을 제공할 수 있었다. 알지네이트 스폰지 및 하이드로젤의 사이토카인 결속효과에 기인하여 대식세포로부터 분비되는 전염증성 사이토카인의 함량이 감소됨을 확인하였으며, 특히 하이드로젤의 사이토카인 억제효과가 더욱 두드러지게 나타났는데, 이는 보다 팽윤된 상태에서 알지네이트 분자의 사이토카인에 대한 결속력이 증가함을 의미한다. 창상형성 초기 하이드로젤에 의한 창상치유 및 수축 효과가 스폰지에 비해 우수한 것으로 나타났으나, 상피화는 스폰지를 적용했을 때 보다 우세하게 진행되었다. 조직학적 평가와 RNA 발현 분석으로부터 알지네이트 스폰지 및 하이드로젤은 혈관 및 콜라겐 섬유의 형성, 상피조직의 재생 및 단백질의 생성 등을 촉진함을 확인하였다.

Abstract: Alginate sponge and hydrogel were prepared by a drying/crosslinking method and their wound healing characteristics were investigated comparatively. The alginate hydrogel had a higher equilibrium water content than the sponge, providing a moist wound healing condition without absorbing exudate from a wound. The amounts of proinflammatory cytokines released by macrophages were lowered due to the cytokine-binding effects of the alginate sponge and hydrogel. The hydrogel lowered the cytokine level more dominantly than the sponge, suggesting that the affinity of alginate molecules to cytokines increases at a more swollen state. The hydrogel allowed superior wound healing and contraction at the early stage of application. However, epithelialization was conspicuous when the sponge was applied. It was confirmed through histological examination and RNA expression analysis that angiogenesis, formation of collagen fibers, regeneration of epithelium, and production of protein were promoted using the alginate sponge and hydrogel as wound dressing materials.

Keywords: alginate, sponge, hydrogel, cytokine, wound healing.

Introduction

A large number of dressings are currently used in the management of burns, split graft donor sites, chronic ulcers, and so on.[1,2] There are two kinds of dressings; dry type and wet type. It has been reported that wounds reepithelialize more rapidly under moist conditions than under dry ones and the rate of dermal repair increases under moist conditions.[3,4]

Sodium alginate, a linear copolymer of 1,4-linked β-D-mannuronate (M) and α-D-guluronate (G) residues, is isolated from marine algae and well dissolved in water due to negatively charged carbonyl group. Alginate is widely used in industry and medicine for many applications such as scaffolds and wound dressings due to low toxicity, favorable mechanical

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properties, and capacity for bioresorption of the constituent materials.[5-8] Alginate dressings are widely used in the treatment of exuding wounds. Alginate maintains a physiologically moist micro environment that promotes healing and the formation of granulation tissue. Alginate can be rinsed away by saline irrigation, thus the removal of the dressing does not interfere with healing granulation tissue. This makes dressing changes virtually painless. Alginate non-woven fabrics are clinically applied for moisture wound healing.

Several types of moist wound dressings are developed for application to variety of wounds. Films, foams, hydrocolloids, hydrogels, and hydrofibers are typical moist wound dressings. Each has different physical property and biological contribution for wound healing. Highly water-soluble sodium alginate is crosslinked with multivalent metal cations, mostly Ca^{2+} ions to produce insoluble calcium alginate in the forms of hydrogels, sponges, sheets, beads, and non-woven fabrics. Though alginate non-woven fabrics were widely and clinically commercialized, they have the current concerns with cytotoxicity and the foreign-body reactions caused by dressing debris.[9]

In this study, the alginate sponge and hydrogel were prepared by a drying/crosslinking method and their physical and biological characteristics including equilibrium water content, cytotoxicity, and proinflammatory cytokine level were evaluated. Finally, comparative study on the wound healing effects of each dressing was carried out with an animal model.

Experimental

Preparation of Alginate Sponge and Hydrogel. One gram of sodium alginate (M_w~500000, FMC Biopolymer, Norway) was dissolved completely in 100 mL of deionized water for an hour. The aqueous sodium alginate solution was poured in a petri dish and freeze-dried for 3 days. The freeze-dried matrix was crosslinked by dipping in 0.2 M $CaCl_2$ solution, washed thoroughly with deionized water, and freeze-dried again to prepare an insoluble alginate sponge. For hydrogel preparation, the aqueous sodium alginate solution was vacuum-dried for a day. The dried matrix was crosslinked by dipping in 0.2 M $CaCl_2$ solution, and washed thoroughly with deionized water.

Equilibrium Water Content. To estimate the free swell absorptive capacity of each sample, the equilibrium water content was measured. The alginate sponge and hydrogel were swollen in excess of deionized water for 72 h at room temperature. The swollen sponge and hydrogel were hung for 30 min to remove free water on the sample surface by gravity. The equilibrium water content was calculated by the following equation: Equilibrium water content (%)=$(M_0-M_d)/M_0\times100$, where M_0 and M_d are the weights of swollen and dried sample, respectively.

Cytotoxicity. Half a gram of the UV-sterilized sample was incubated in 50 mL of dulbecco's modified eagle's medium (DMEM) (HyClone, USA) at 37 °C for 24 h under shaking. Afterwards, the extract was filtered to remove insoluble material residues and sterilized by passage through a 0.2 mm filter. Primary-cultured human fibroblast cells (passage number: 5), which had been previously isolated from human skin provided by the department of plastics and reconstructive surgery, Kyungpook National University Hospital, Korea, were cultured in DMEM supplemented with 1% penicillin and 10% fetal bovine serum. The cells were cultured at 37 °C in 5% CO_2 atmosphere for 3-5 days and 1.0×10^6 cells were seeded into each well of 96-well culture plates. After 24 h, the culture medium was replaced by either fresh DMEM or the extract. Cells were then further incubated for 24 h. After replacing the old medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA) solution (5.0 mg/mL) was added to each well, and the cells were incubated for 4 h. The cell viability was obtained from the degree of mitochondrial reduction of MTT to formazan by succinic dehydrogenase. The absorbance at 570 nm was measured using a microplate reader (Molecular devices, USA). Cell viability (%) was expressed as the relative absorbance of the sample to that of the control (means±SD). Differences were considered statistically significant at a level of $p<0.05$.

Proinflammatory Cytokine Level. RAW 264.7 cells (murine macrophage cell line, Korean Cell Line Bank) were cultured in the growth medium at 37 °C. When the cells reached 80% confluence, they were trypsinized with 0.25% trypsin containing 1 μM ethylenediaminetetraacetic acid (Gibco, USA) and counted by a hemacytometer (Hausser Scientific, USA) prior to further use. Then the cells (1.0×10^5) were cultured and stimulated in 1.5 mL DMEM supplemented with 1% penicillin, 10% fetal bovine serum and 1.0 μg/mL of lipopolysaccharide with the sample (diameter: 0.5 cm and thickness: 0.1 cm) for 24 h. The pro-inflammatory cytokine level of each culture supernatant was quantified using an enzyme-linked immunosorbent assay kit (R&D system, USA) according to the manufacturer's protocol. Cytokine (%) was expressed as the relative absorbance of the sample to that of the control (means±SD). Differences were considered statistically sig-

nificant at a level of $p<0.05$.

In vivo Wound Healing Test. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Kyungpook National University (Permit Number: 2013-0042). All animal experiments were performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and the guidelines of IACUC. Male Sprague-Dawley (SD) rats (6 weeks old, 200~250 g) were used to evaluate the wound healing characteristics of sponge and hydrogel. Full thickness skin wound of 1.5 cm ×1.5 cm area was made on the back of rats. In order to induce anesthesia during the incision, 10 mg/kg of xylazine hydrogen chloride (Rompun, Bayer Korea, Korea) and 100 mg/kg of ketamine hydrochloric acid (Ketamine, Huons, Korea) were mixed and injected into the abdominal cavity of rats. After the rats were anesthetized, they were prone positioned. Then the hair on the back of rats was shaved and sterilized using povidone-iodine and 70% alcohol. After making incisions with a No. 15 surgical blade, the muscular and subcutaneous layers were separated using a Metzenbaum scissors, and whole-layer skin wounds were made. After forming wounds, gauze (control) and the samples were applied on the wound sites. 12 rats were used for each group. Finally, the wounds were covered with gauze and fixed lightly using a 2-inch elastic support bandage.

Evaluation of Wound Healing. For gross examination of wound, the Visitrak Grid films and the Visitrak Digital wound analysis system (Smith & Nephew, UK) were used. The border of whole-layer wound which was made right after the operation was traced on a sterile, transparent film, and the corresponding area was labeled W_0. After a few days, granulomatous tissue was formed in the central area of the wound. The outer area of wound that was healed due to epithelialization was traced and labeled W_i, and the actual area of granulomatous tissue was labeled U_i (Figure 1). After a digital photo was taken, each wound was drawn on the Visitrak Grid film and the whole wound and epithelialized areas were measured. The percentages of wound contraction, wound healing, and wound epithelialization were calculated according to the following equations: Wound healed (%)=$(W_0-U_i)/W_0\times100$, Wound contraction (%)=$(W_0-W_i)/W_0\times100$, Wound epithelialization (%)=$(W_i-U_i)/W_0\times100$.

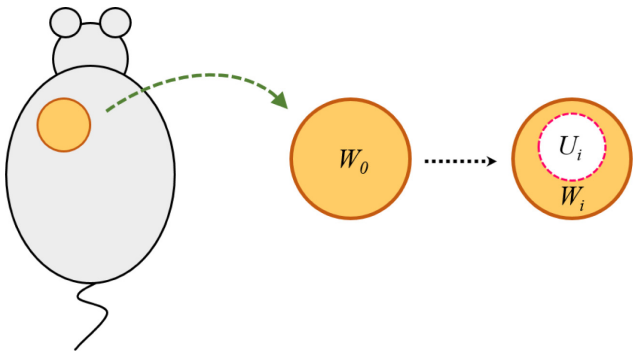

Figure 1. W_0, W_i, and U_i measured from the whole wound and epithelialized areas in a rat.

Histology. The wound tissue was extracted from the section of wound margin including normal skin, and fixed with 3.7% formaldehyde for 1 h. Then, the fixed tissue was embedded with paraffin. For Hematoxylin and Eosin (H&E) staining, the paraffin-embedded tissues were sectioned and stained with Mayer's hematoxylin for 15 min and washed in running tap water. Counterstaining with eosin was performed for 15 s~2 min depending on the age of the eosin and the depth of the counterstain desired. And then the wound tissues were dehydrated with 95% alcohol until excess eosin was removed. Lastly, the tissues were washed with xylene and mounted in slides. For Masson's trichrome staining, the fixed tissues were stained in Weigert's iron hematoxylin working solution for 10 min. After rinsed in running warm tap water, the wound tissues were stained in Biebrich scarlet-acid fuchsin solution for 10~15 min and differentiated in phosphomolybdic-phosphotungstic acid solution for 10~15 min. Afterward, the tissues were transferred directly to aniline blue solution, stained for 5~10 min, and differentiated in 1% acetic acid solution for 2~5 min. Finally, the stained wound tissues were dehydrated very quickly with 95% ethyl alcohol, absolute ethyl alcohol, and cleared in xylene. Under a light microscope, the inflammatory reaction of the wound site, vascularization, collagen fiber formation and arrangement, and the regeneration of epithelia were observed.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). RT-PCR was performed for analysis of mRNA expression of skin wounds of rats. RNA was separated using RNA isolation kit (Invitrogen, USA). One milligram of extracted RNA was reversely transcribed to complete DNA (cDNA) using reverse transcriptase. Polymerase chain reaction of reversely transcribed cDNA was performed using GeneAmp RNA PCR kit (Takara, Japan). In our experiment, the genes which was amplified using RT-PCR were glyceraldehydes-3-phosphate dehydrogenase (GAPDH), vascular endothelial growth factor (VEGF), and transforming growth factor β (TGF-β). GAPDH was a housekeeping gene which was used

as a control for normalization of the loading volumes to produce equal levels of expression. The RT-PCR reactions were performed through 28 cycles of denaturation (94 ºC, 30 s), annealing (60 ºC, 30 s), and extension (72 ºC, 30 s) for gene amplification. After stabilization at 72 ºC for 5 min, the amplified cDNA was separated on a 1.5% agarose gel containing 1.0 mg/mL ethidium bromide (Sigma, USA). The gel was scanned and its image was captured by LAS4000 (Fujifilm, Japan).

Results and Discussion

Sodium alginate, a sodium salt of alginic acid, is soluble in water and has the unique property of easily forming a gel in the presence of multivalent metal cations such as Ca^{2+}, Cu^{2+}, and Zn^{2+}. Therefore, alginate can be easily processed into various shapes through its gelation property. However, simple addition of aqueous $CaCl_2$ solution to sodium alginate solution results in a heterogeneous sol/gel mixture including insoluble precipitates, which is hardly fabricated to a construct with a desired shape. For this reason, we prepared the sheet type alginate sponge and hydrogel by the drying/crosslinking method. Through the drying step, a sodium alginate sheet was produced. Afterward, the dried sodium alginate sheet was dipped in an aqueous $CaCl_2$ solution to prepare a calcium alginate sheet. The appearance of a calcium alginate construct depended on the drying condition and shape of a mold. For the sponge preparation, two step freeze-drying (freeze drying/crosslinking/freeze-drying) was applied. The resulting calcium alginate sponge was highly porous and able to absorb a large amount of water. As shown in Table 1, the alginate sponge had a high free swell absorptive capacity, suggesting that it is applicable to the wound with a lot of exudate. In this case, the moist wound healing can be conducted by absorbing exudate. On the other hand, the alginate hydrogel sheet was produced from a vacuum-dried sodium alginate sheet film. In its crosslinking process, calcium alginate formation took place first on the surface of film and the migration of calcium ions was limited. On the other hand, a freeze-dried sodium alginate sheet had a lot of pores to provide a better condition for Ca^{2+} migration. It is considered that the alginate hydrogel had more uncrosslinked sodium alginate moieties inside than the sponge, which is responsible for a higher equilibrium water content. In spite of this, the hydrogel is not able to absorb exudate as much as the sponge because it is a water-containing wound dressing material. However, the hydrogel can provide a moist wound healing condition without absorbing exudate from a wound.

Table 1. Equilibrium Water Contents of the Alginate Sponge and Hydrogel

	Water content (%)
Alginate sponge	92.5
Alginate hydrogel	95.5

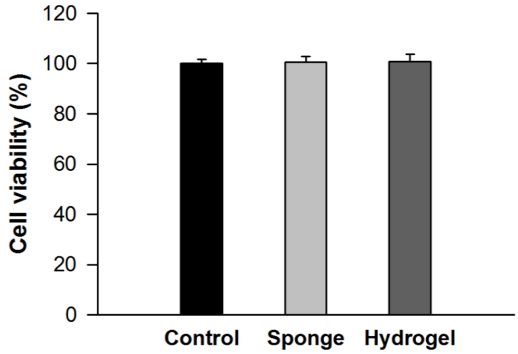

Figure 2. Relative viability of human fibroblasts treated with the extracts of alginate sponge and hydrogel (n=3, p>0.6).

It is well known that alginate is a biocompatible polymer and the treatment of cells with the extracts of alginate dressings had little negative influence on cell viability and cell proliferation.[10] Figure 2 shows the extract of alginate sponge and hydrogel did not affect the viability of human fibroblasts. The extraction was carried out using a cell culture medium. The alginate sponge and hydrogel could be dissolved in part during the extraction process because some crosslinking calcium ions were possibly exchanged by sodium ions in the media. Although free alginate molecules are considered to have no cytotoxicity at all, it should be noted that cytotoxic NaCl can be formed while crosslinking sodium alginate with $CaCl_2$. In order to minimize the cytotoxicity of a calcium alginate construct, complete washing is essential right after crosslinking procedure.

The wound healing process is separated into three overlapping phases: (1) inflammation, (2) re-epithelialization and granulation tissue formation, and (3) matrix formation and remodeling.[11,12] It has long been speculated that proinflammatory cytokines play an important role in wound repair. The invasion of monocytes into the wound tissue and their differentiation into macrophages are essential for normal repair.[13] Activated macrophages can produce several proinflammatory cytokines, including interleukins 1 alpha and beta (IL-1α and IL-1β),[14] interleukin 6 (IL-6),[15] and tumor necrosis factor alpha (TNF-α).[16,17] These cytokines exert a series of biological activities which might be important for wound healing. IL-1 is a pleiotropic proinflammatory cytokine responsible for funda-

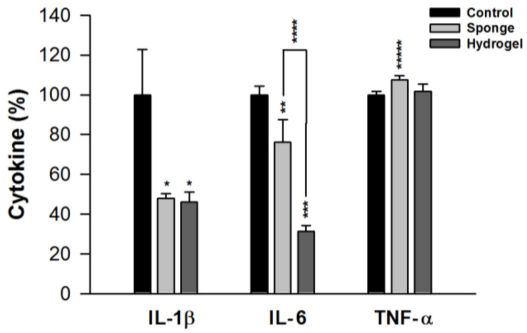

Figure 3. Relative amount of proinflammatory cytokines released from RAW 264.7 cells cultured with the alginate sponge and hydrogel (n=3, *p<0.02, **p<0.03, ***p<0.00005, ****p<0.005, *****p<0.01).

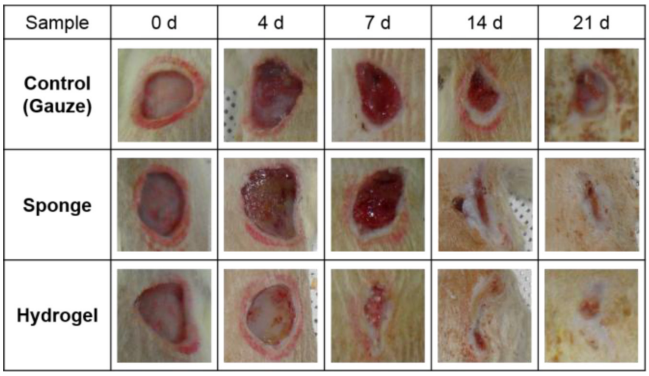

Figure 4. Photographs of the wounds in the control (gauze), the alginate sponge, and the hydrogel groups.

mental functions in wound healing, inflammation, and host antitumor responses.[18] A small amount of IL-1 is necessary for host defense and wound healing, whereas overproduction of IL-1 can hinder the early phase of wound healing.[19,20] TNF is another central mediator of inflammatory responses, playing important roles in antimicrobial defense, wound healing, and defense against malignant disorders.[21] Although small amounts of TNF are necessary for host defense against infection, overproduction of TNF can be detrimental. TNF-α, associated with chronic inflammation, is secreted by macrophages and mast cells. The levels of these cytokines are profoundly elevated in chronic wounds.[22-24] It was reported that alginate is able to bind proinflammatory cytokines in a time-dependent manner.[10] For this reason, the amounts of IL-1β and IL-6 released by macrophages were lowered as shown in Figure 3. Each alginate construct, as a foreign body, may induce macrophage cells' inflammatory reaction and subsequent cytokine release. However, this can be inhibited by the cytokine-binding effect of alginate. The hydrogel lowered the IL-6 level more dominantly than the sponge, suggesting that the affinity of alginate molecules to IL-6 increases at a more swollen state. The cytokine inhibition activity of alginate sponge and hydrogel is considered to reduce inflammation due to foreign body reaction as a wound dressing or an implant material. The sponge and hydrogel are regarded as good candidates to make up for the drawbacks of alginate non-woven dressing. On the other hand, the level of TNF-α was slightly increased. The cytokine-binding effect of alginate was not sufficient enough to reduce the amount of TNF-α released from macrophage cells.

For a comparative study on the wound healing using alginate sponge and hydrogel, they were applied to the fresh wounds of SD rats. The wounds contracted accompanying epithelialization as time passed, with each experimental group showing different appearances. As wound contraction and epithelialization progressed simultaneously, the non-epithelialized granulomatous tissue decreased, and wound healing was observed. No experimental group showed any signs of infection, and all groups had a small amount of fluid issuing from the wound up until the seventh day after the injury. Figure 4 shows the photographs of wounds changed with time. On the 4th d after surgery, granulation tissues were observed. On the 7th day postoperatively, the size of wounds was reduced significantly especially for the alginate hydrogel and epithelialization started in both experimental group. It is obvious that the sponge and the hydrogel promoted wound healing remarkably as compared with the control, gauze.

Based on the progress of wound healing over time with the application of each sample, the hydrogel and the sponge applied groups showed a significantly higher percentage of wound healing than the control group (Figure 5). Full-thickness skin wounds are healed through wound shrinkage and epithelialization. It is notable that the hydrogel revealed a superior wound healing due to a high degree of wound shrinkage on the 4th and 7th days. Exudate was not seriously formed in the fresh wound site of our animal model. The alginate hydrogel, a water-containing material, was able to provide a sufficient moist wound healing condition by itself and most effective in wound healing at the early stage of application. The alginate sponge could have a gel-like swollen region, resulting from partial exchange of crosslinking Ca^{2+} with Na^{+} by absorbing exudate. For this reason, moist wound healing was achieved using the alginate sponge. After 14 days, the wound healed percentages of hydrogel and sponge groups were almost same. Similar trend was found for wound contraction as shown in

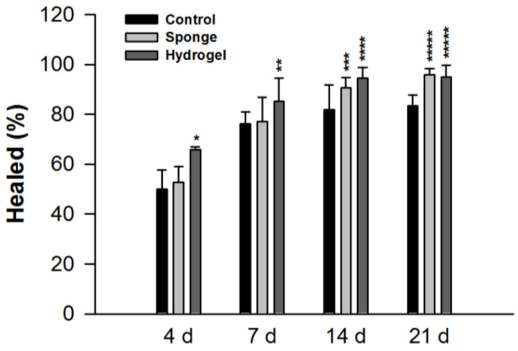

Figure 5. Wound healed (%) of the control (gauze), the sponge, and the hydrogel groups (n=3, *p<0.15, **p<0.25, ***p<0.25, ****p<0.15, *****p<0.05).

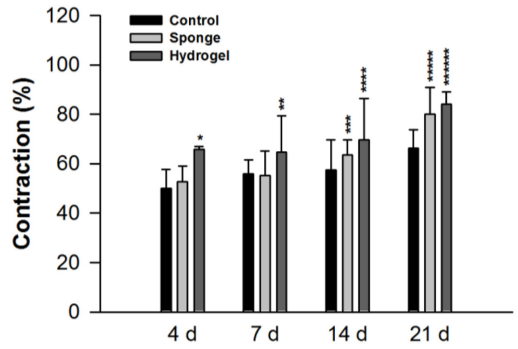

Figure 6. Wound contraction (%) of the control (gauze), the sponge, and the hydrogel groups (n=3, *p<0.15, **p<0.4, ***p<0.5, ****p<0.4, *****p<0.2, ******p<0.05).

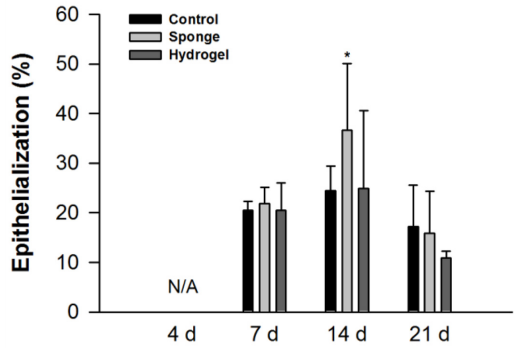

Figure 7. Wound epithelialization (%) of the control (gauze), the sponge, and the hydrogel groups (n=3, *p<0.25).

Figure 6. On the other hand, epithelialization was conspicuous when the alginate sponge was applied especially on the 7th and 14th days as shown in Figure 7. The sponge promoted wound shrinkage and contraction less actively than hydrogel, resulting in the higher values of W_i, W_i–U_i, and would epithelialization (%). On the contrary, the W_i for the hydrogel group decreased

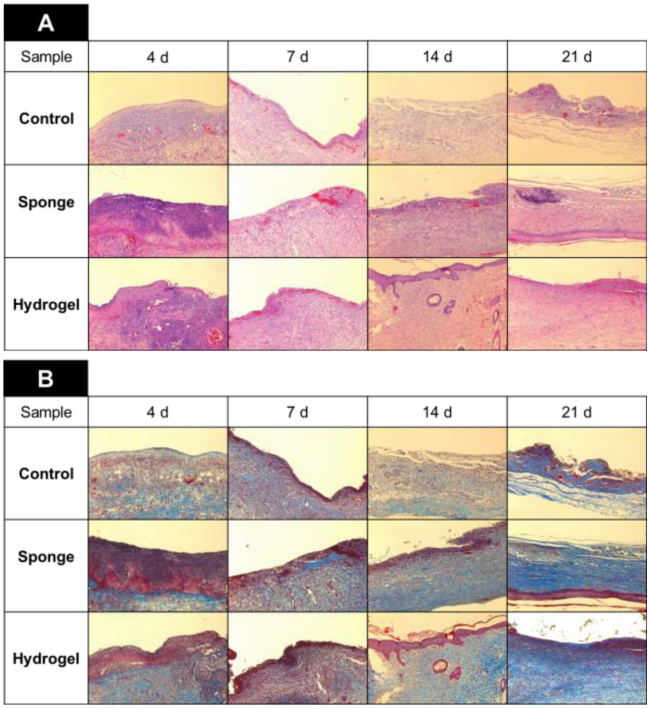

Figure 8. Histology for wound tissues of the control (gauze), the sponge, and the hydrogel groups (A: H&E staining, B: Masson's trichrome staining).

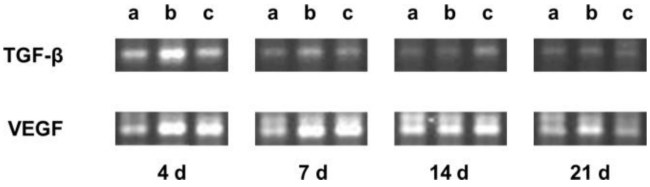

Figure 9. RT-PCR analysis of TGF-β and VEGF for the control (gauze) (a); the sponge (b); the hydrogel (c) groups.

rapidly to lower the W_i–U_i. In this case, granulation prevailed over epithelialization with rapid wound shrinkage and contraction.

Up until the 7th day after the injury, polymorphonuclear cells, neutrocytes and lymphocytes, indications of inflammation, were seen in every experimental group (Figure 8(A)). Also, higher degree of angiogenesis and more condensed, regular arrangements of collagen fibers were seen in the alginate sponge and hydrogel groups on 14th day (Figure 8(B)). When the wounds were completely healed, thicker, more matured, and more regenerated epithelia were found in the alginate sponge and hydrogel groups. In the RT-PCR experiment, the expressions of protein such as TGF-β and VEGF for the alginate sponge and hydrogel groups were much higher than those

for the control as shown in Figure 9. The sponge showed stronger expressions on the 4th day. VEGF has strong stimulating effects on vascularization and promotes wound healing.[25] TGF-β has direct stimulatory effects on the synthesis of extracellular matrix proteins and also increases the number of endothelial cells by its angiogenic effects resulting in the formation of new granulation tissue. TGF-β has several properties suggesting the ability to enhance wound repair.[26] The alginate sponge and hydrogel are considered to activate the protein productions which are required for tissue regeneration and wound healing.

Conclusions

In this study, the alginate sponge and hydrogel were prepared and their wound healing characteristics were investigated. The hydrogel is considered to provide a better moist wound healing condition than the sponge because it is a water-containing material and has a higher equilibrium water content. Each alginate construct inhibited the proinflammatory cytokines such as IL-1β and IL-6 released by macrophage cells. The hydrogel lowered the IL-6 level more dominantly than the sponge, suggesting that the affinity of alginate molecules to IL-6 increases at a more swollen state. The hydrogel allowed a superior wound healing and contraction especially at the early stage of application. However, epithelialization was not so active as in the case of sponge due to rapid wound shrinkage. Higher degree of angiogenesis, more condensed and regular arrangements of collagen fibers, thicker and more matured epithelium regeneration, and higher expression of TGF-β and VEGF were confirmed for the alginate sponge and hydrogel groups, which is responsible for their excellent wound healing ability.

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