



Soft Tissue Augmentation with Silk Composite Graft

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Abstract

Purpose: The objective of this study was to evaluate the interaction between 4-hexylresorcinol (4HR) and antibody as that affects the performance of a silk-4HR combination graft for soft tissue augmentation in an animal model.

Methods: The silk graft materials consisted of four types: silk+10% tricalcium phosphate (TCP) (ST0), silk+10% TCP+1% 4HR (ST1), silk+10% TCP+3% 4HR (ST3), and silk+10% TCP+6% 4HR (ST6). The antibody binding assay tested the 4HR effect and scanning electron microscopic (SEM) exam was done for silk grafts. The animal experiment used a subcutaneous pocket mouse model. The graft - SH0 or SH1 or SH3 or SH6 - was placed in a subcutaneous pocket. The animals were killed at one, two, and four weeks, postoperatively. The specimens were subjected to histological analysis and lysozyme assay.

Results: Groups with 4HR applied showed lower antibody binding affinity to antigen compared to groups without 4HR. In the SEM examination, there was no significant difference among groups. Histological examinations revealed many foreign body giant cells in ST0 and ST1 group at four weeks postoperatively. Both ST3 and ST6 groups developed significantly lower levels of giant cell values compared to ST0 and ST1 groups ($P < 0.001$) at four weeks postoperatively. In the lysozyme assay, the ST1 and ST3 groups showed denser signals than the other groups.

Conclusion: 4HR combined silk implants resulted in high levels of vascular and connective tissue regeneration.

Key words: Composite tissue allografts, Silk, Hexylresorcinol, Mice, Metabolism

Introduction

Soft tissue defects in the facial area result from conditions such as trauma, cancer, and infection. As the facial morphology is important to personal identity, facial defects cause not only functional problems but also social and psychological problems. Given the importance of aesthetic reconstruction for facial defects, volumetric restoration should use the most effective graft material[1,2]. For the reconstruction of facial soft tissue defects, many different

treatments are available. Most facial soft tissue defects cannot be treated by simple bone augmentation, and must be accompanied by soft tissue transfer or graft[3]. Large defects can be successfully treated by local flap[4] or microvascular flap[2]. Many types of free grafts are used for small defect.

Free graft materials for the soft tissue augmentation can be autogenous, allogenic, xenogenic, or alloplastic. Among the autogenous materials, free fat graft[5] and dermal graft[6] are most popular. However, complications such

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as postoperative infection, donor site morbidity, and excessive resorption are reported[7,8]. Allogenic or xenogenic collagen are also popular for this purpose[9-11], but these materials have drawbacks such as disease transmission and immune reaction[12,13].

Silk is produced by an insect and is mainly composed of fibroin and sericin. It has been used as a suture material for centuries. When it is used to tie vessels, it can be implanted into the body without serious problems. Silk fibroin has recently been examined as a scaffold in the tissue engineering[14,15]. Recent applications of silk based biomaterials include tympanic membrane[16], bone graft[14], and burn dressing[15]. As silk is slowly biodegradable, it can be used as a membrane for guided bone regeneration[17]. Its low degradability makes it useful as soft tissue augmentation material. However, many foreign body giant cells are involved in silk degradation[18,19]. This foreign body reaction may lead to chronic inflammation.

4-Hexylresorcinol (4HR) is a molecular chaperone of bacterial origin[20]. It is used as an antiseptic[21] and food additive[22]. 4HR also has an anti-cancer effect and inhibits the nuclear factor- κ B pathway[23]. 4HR combined with hydroxyapatite is used as bone graft material[24]. Silk with 4HR combination graft performs better than silk without 4HR[25]. 4HR can inhibit giant cell formation induced by silk fibroin via the diacylglycerol kinase pathway[25]. Additionally, 4HR can inhibit antibody binding capacity[26]. Therefore, silk with 4HR combination graft may perform better when used for soft tissue augmentation.

In this study, the inhibition of antibody binding capacity by 4HR was re-evaluated. For soft tissue augmentation material, 4HR at differing concentrations was mixed with silk fibroin. Performance was evaluated in the mouse back skin pouch model. The objective of this study was to examine the interaction between 4HR and antibody and the performance of silk-4HR combination graft for soft tissue augmentation in the animal model.

Materials and Methods

1. Preparation of graft material

The silk graft materials were kindly donated by the Rural Development Administration (Suwon, Korea). Silk fibroin

macromolecules without sericin were dissolved in hydrochloric acid, and an electro dialysis system was used to remove salt. It was made into four types of graft materials: silk+10% tricalcium phosphate (TCP) (ST0), silk+10% TCP+1% 4HR (ST1), silk+10% TCP+3% 4HR (ST3), and silk+10% TCP+6% 4HR (ST6).

2. Antibody binding assay

The antibody binding assay was referenced from a previous publication. The experiments were carried out using the Anti-*Toxoplasma gondii* immunoglobulin G Human ELISA kit (ab108776; Abcam, Burlingame, CA, USA) for quantitative enzyme immunoassay of immunoglobulin G specific for *T. gondii*. Control specimens from kits with known activity measured in international units (ME/mL) were used as antibody sources.

3. Scanning electron microscopic exam

All materials were prepared for scanning electron microscopic (SEM) examination. Subsequent procedures were undertaken by Gangneung Center of Korea Basic Science Institute. After immobilization of the samples on the plate, each sample was coated with gold and examined by scanning electron microscopy (H-800; Hitachi, Tokyo, Japan).

4. Animal experiment

Sixty 8-week-old mice were used in this experiment. This experiment was approved by the Institutional Animal Care and Use Committee of Gangneung-Wonju National University, Gangneung, Korea (No. 2010-16).

General anesthesia was induced by intramuscular injection of a combination of 120 mg/kg of ketamine (Ketara; Yuhan, Seoul, Korea) and 2 mg/kg of xylazine (Rompun; Bayer Korea, Seoul, Korea). The back skin area was shaved and disinfected with povidine-iodine. After a longitudinal incision, subcutaneous dissection created a 1-cm long subcutaneous pocket. The graft - SH0 or SH1 or SH3 or SH6 - was placed in the pocket. The volume of each graft was 0.5 mL. The assignment for graft type was random with fifteen mice in each group. The incision was closed in layers with 3-0 silk. Twenty animals were humanely sacrificed at one week, 20 at two weeks, and 20 at four weeks. The dimension of the skin specimens was approximately 15×10×3 mm. They were fixed in 10% formalin. The speci-

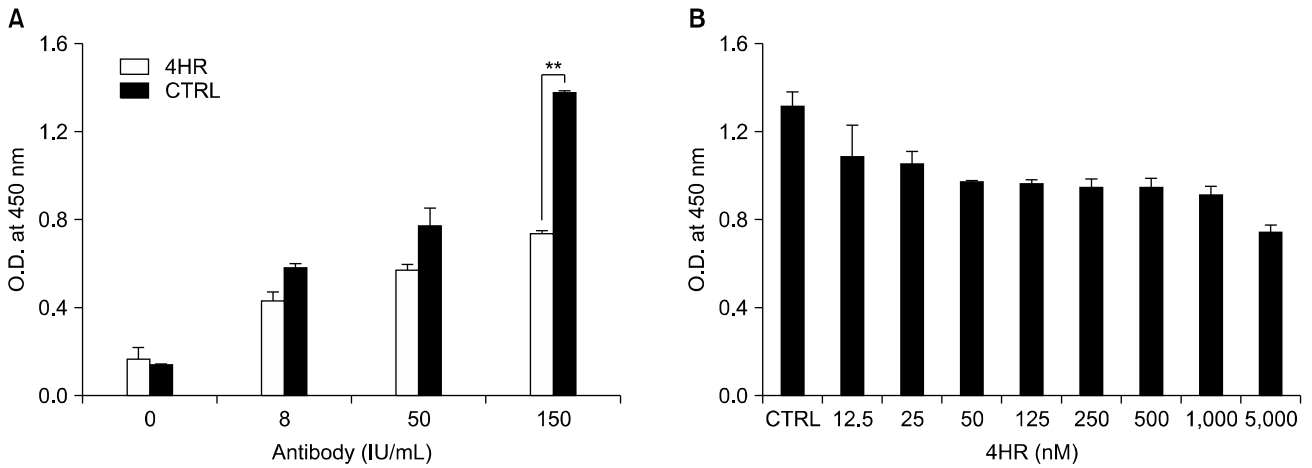


Fig. 1. The antibody binding assay. (A) As the quantity of antibody increases from 0 to 150 IU/mL, 1 μ M 4HR groups showed significantly lower antibody binding than the groups without 4HR (** $P < 0.001$). (B) When applied antibody quantity was fixed at 150 IU/mL, the antibody binding to the antigen decreased to the applied 4HR concentration dose-dependently. 4HR, 4-hexylresorcinol; CTRL, control without 4HR; O.D., optic density.

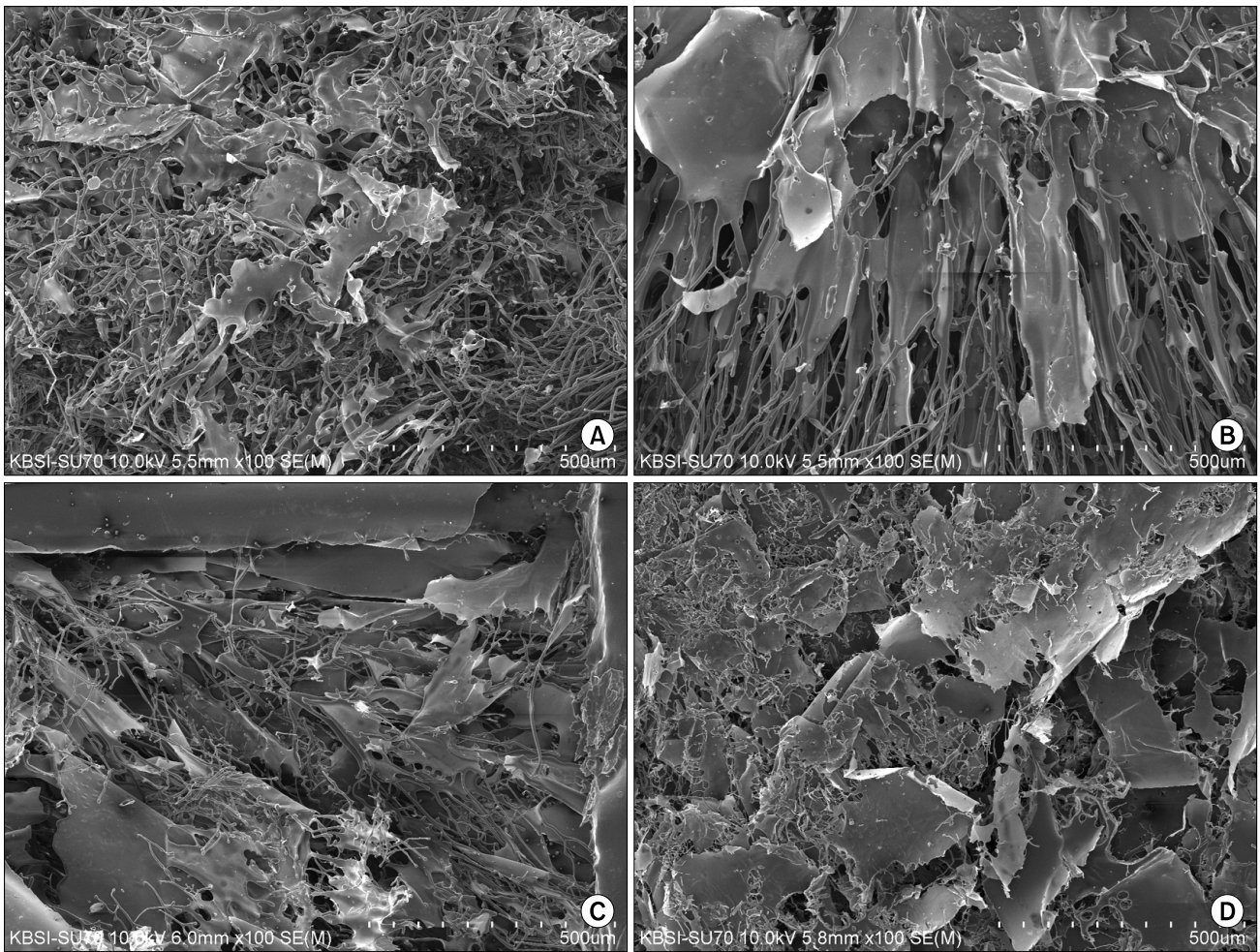


Fig. 2. Scanning electron microscopic examination ($\times 100$). (A) Silk+10% tricalcium phosphate (TCP), (B) silk+10% TCP+1% 4-hexylresorcinol (4HR), (C) silk+10% TCP+3% 4HR, and (D) silk+10% TCP+6% 4HR.

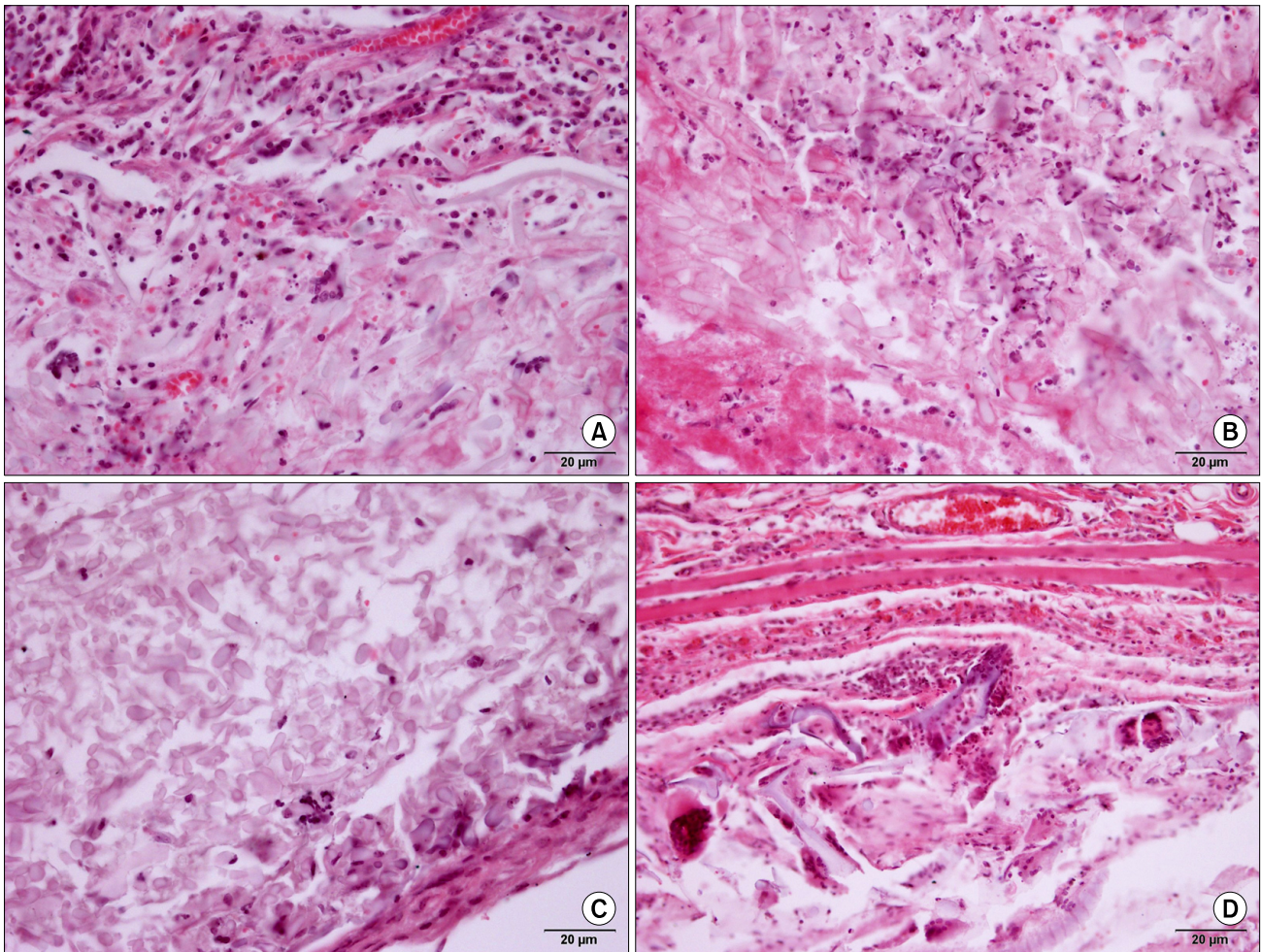


Fig. 3. Histological examinations at one week postoperatively (H&E, $\times 200$). (A) Silk+10% tricalcium phosphate (TCP), (B) silk+10% TCP+1% 4-hexylresorcinol (4HR), (C) silk+10% TCP+3% 4HR, and (D) silk+10% TCP+6% 4HR.

mens were dehydrated and embedded. A segment was embedded as sagittal sections in paraffin blocks. The paraffin blocks were sliced and stained with hematoxylin and eosin to evaluate newly regenerated connective tissue and foreign body reaction. Foreign body reaction was evaluated for each group by counting the number of nuclei in representative power microscopic fields (150~200 cells) and recording the fraction of nuclei within multinucleated cells (cells with >2 nuclei). The lysozyme assay was done with anti-lysozyme antibody (sc-27956; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The subsequent procedure followed conventional immunohistochemical analysis.

5. Statistical analysis

The independent samples t-test was used to compare difference between two independent groups. For comparison

of three independent groups, ANOVA test was used. *Post hoc* testing used the Bonferroni method. Null hypotheses of no difference were rejected if *P*-values were less than 0.05.

SPSS statistics ver. 14 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis.

Results

In the antibody binding assay, 4HR application groups showed lower antibody binding affinity to antigen when compared to the groups without 4HR (Fig. 1). As the quantity of antibody increased from 0 to 150 IU/mL, 1 μ M 4HR applied groups showed significantly lower antibody binding than the groups without 4HR ($P < 0.001$; Fig. 1A). When the antibody quantity was fixed at 150 IU/mL, the antibody binding to the antigen decreased to the 4HR con-

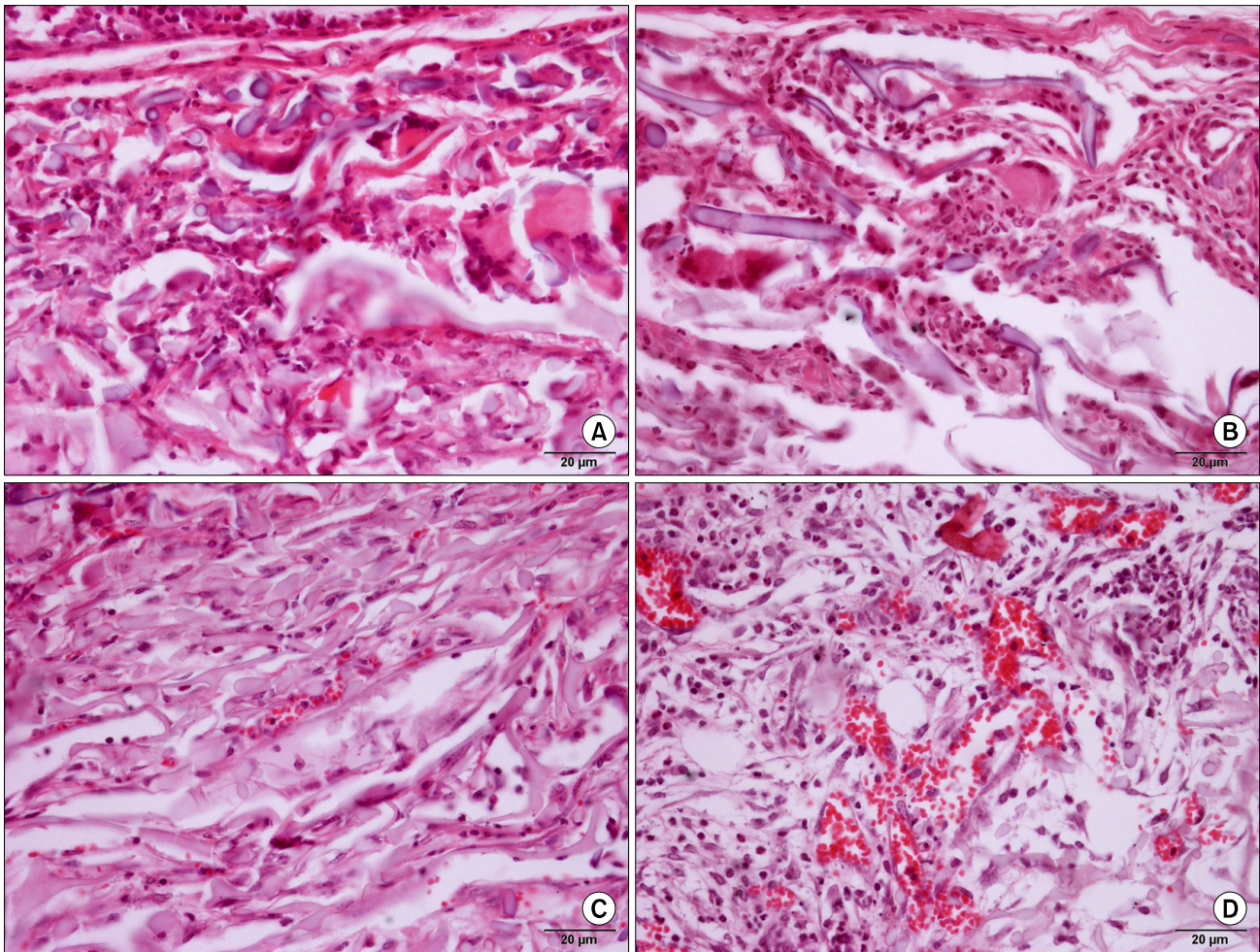


Fig. 4. Histological examinations at two weeks postoperatively (H&E, $\times 200$). (A) Silk+10% tricalcium phosphate (TCP), (B) silk+10% TCP+1% 4-hexylresorcinol (4HR), (C) silk+10% TCP+3% 4HR, and (D) silk+10% TCP+6% 4HR.

centration dose-dependently (Fig. 1B).

In the SEM examination, the specimen without 4HR showed many string-like structures (Fig. 2). The specimens with 4HR showed more sheet-like structures than the group without 4HR. However, the differences among groups were not significant.

Histological examinations revealed the formation of inflammatory cells in all groups at one week postoperatively (Fig. 3). However, ST3 groups showed low level of cellular infiltration into the implant. ST0 group and ST1 group showed prominent giant cell formation at two weeks postoperatively (Fig. 4). ST3 group developed vascular networks with fibrotic tissue ingrowths. ST6 group had many more vascular networks than other groups at two weeks postoperatively. Many foreign body type giant cells were observed in ST0 and ST1 group at four weeks post-

operatively (Fig. 5). ST3 and ST6 group also showed foreign body giant cells, but the number of giant cells was less than ST0 and ST1 group. Giant cell formation was inhibited by 4-HR administration (Fig. 6). The percent of giant cells in ST0 was $49.6\% \pm 2.1\%$, while ST1 was $46.8\% \pm 6.0\%$, ST3 was $12.2\% \pm 1.4\%$, and ST6 was $22.8\% \pm 2.0\%$ (Fig. 6). The differences among groups were statistically significant ($P < 0.001$). In the post hoc test, both ST3 and ST6 groups had significantly lower values compared to ST0 and ST1 groups ($P < 0.001$). ST3 group had significantly lower cell fusion than the ST6 group ($P = 0.001$). Well-organized vascular network and fibrotic tissue were observed in ST3 group at four weeks postoperatively. The ST6 group also showed newly formed fibrotic tissue, although the density of fibrotic tissue was less than ST3 group. In the lysozyme assay, ST1 and ST3 group showed dense signal

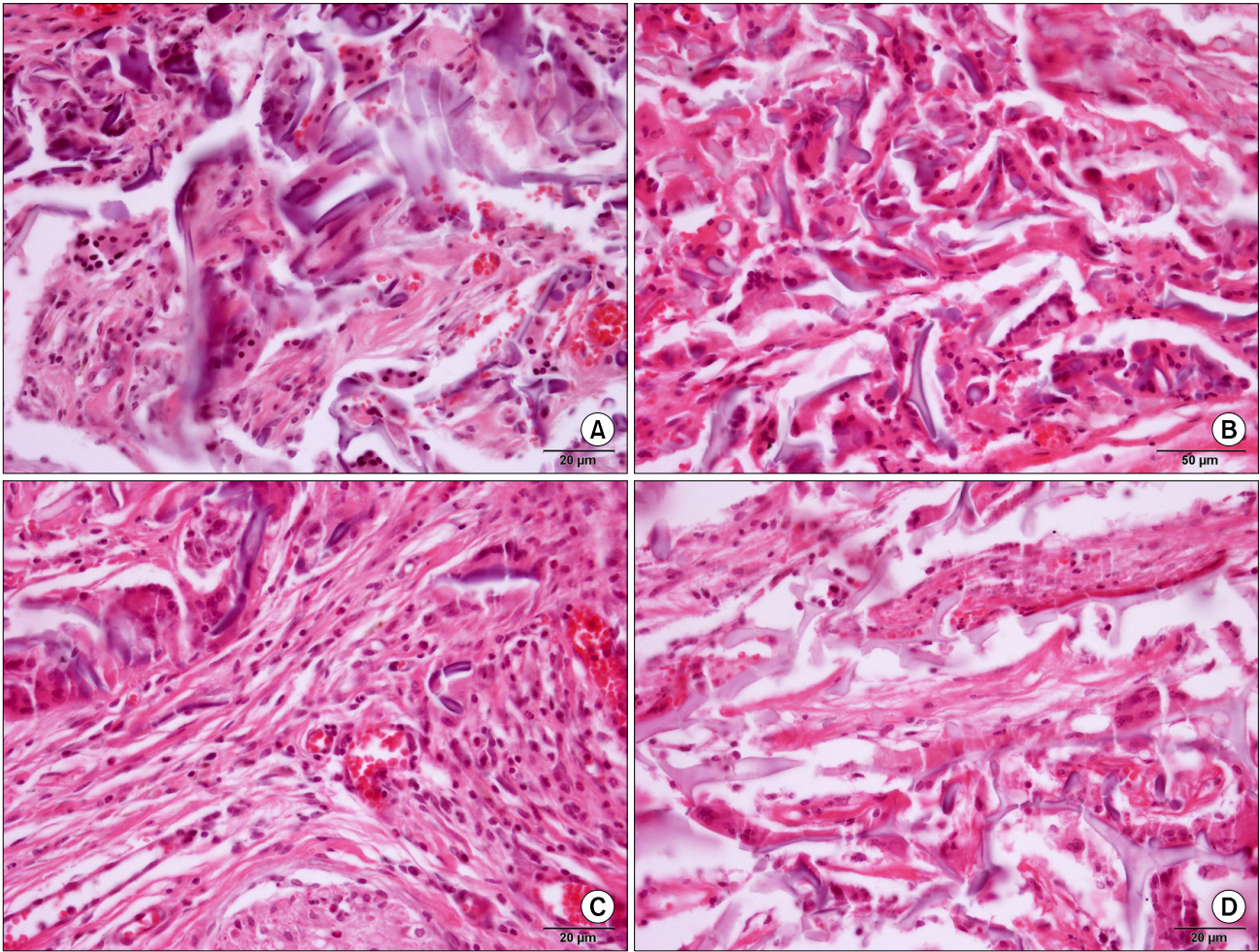


Fig. 5. Histological examinations at four weeks postoperatively (H&E, x200). (A) Silk 10% tricalcium phosphate (TCP) (ST0), (B) silk+10% TCP+1% 4-hexylresorcinol (4HR) (ST1), (C) silk+10% TCP+3% 4HR (ST3), and (D) silk+10% TCP+6% 4HR (ST6). (A, B) Many foreign body giant cells were observed in ST0 and ST1 group at four weeks postoperatively. (C, D) ST3 and ST6 group also showed foreign body giant cells, but the number of giant cells was less than ST0 and ST1 group.

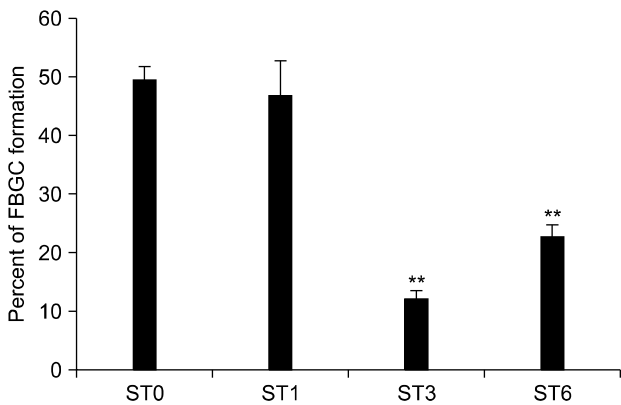


Fig. 6. The percent of giant cell (ST0: silk+10% tricalcium phosphate (TCP), ST1: silk+10% TCP+1% 4-hexylresorcinol (4HR), ST3: silk+10% TCP+3% 4HR, and ST6: silk+10% TCP+6% 4HR) (** $P < 0.001$). FBGC, foreign body giant cell.

than the other groups (Fig. 7). However, ST3 group showed more localized signal than ST1 group.

Discussion

The reconstruction of soft tissue defects is challenging for the reconstructive surgeon. The main reason for unpredictable results is the implant material. Autogenous implants such as dermis and fat have a high resorption rate[27]. Non-autogenous implants have a high rate of complications such as postoperative infection and avascular necrosis[12,13]. In this study, 4HR combination silk implants yielded a high level of vascular and connective tissue regeneration. To the best of our knowledge, this has not been reported.

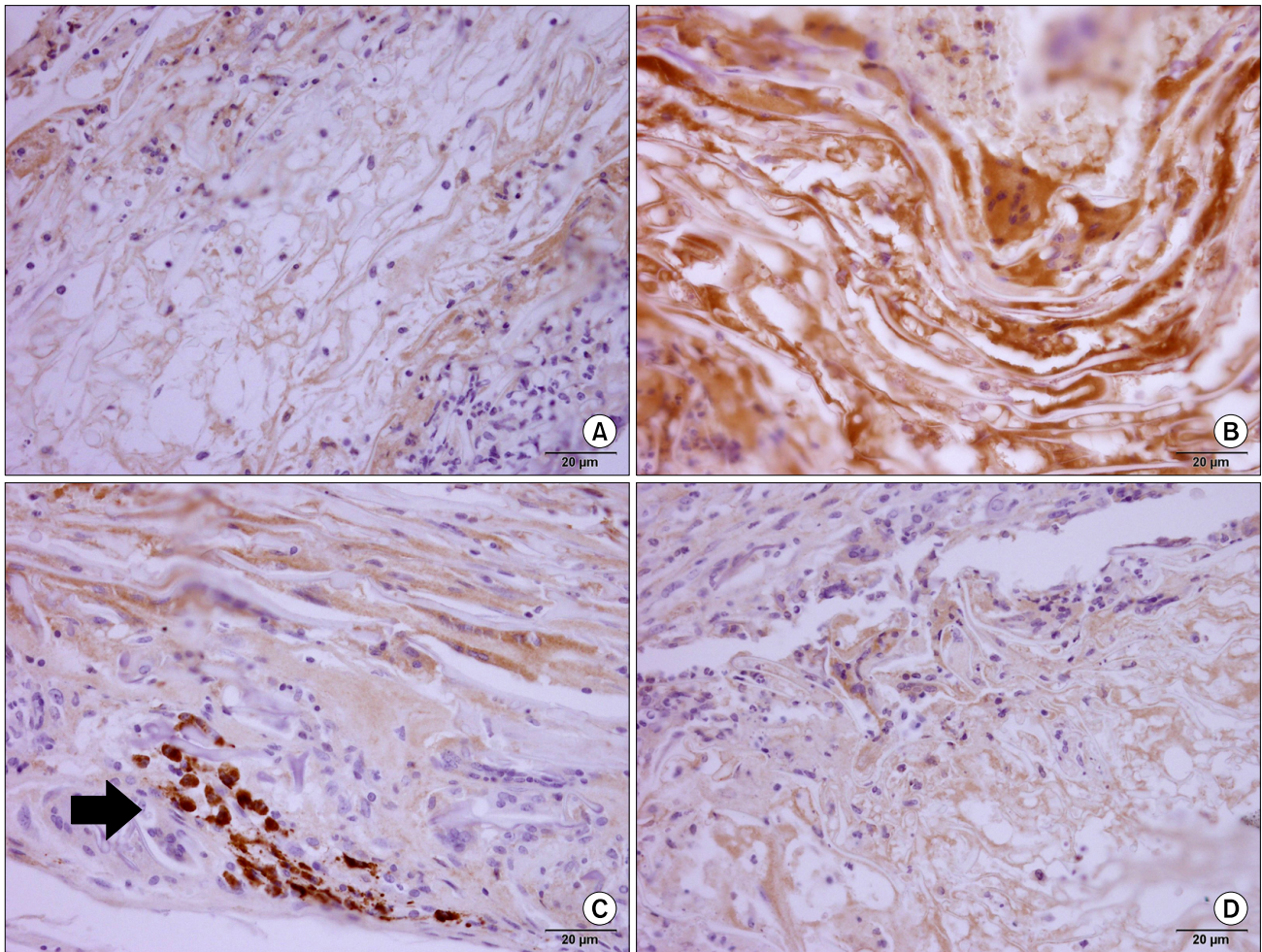


Fig. 7. Lysozyme assay (Counterstained by Mayer's hematoxyline, $\times 200$). A: silk+10% tricalcium phosphate (TCP), B: silk+10% TCP+1% 4-hexylresorcinol (4HR), C: silk+10% TCP+3% 4HR, D: silk+10% TCP+6% 4HR. The groups having 1% and 3% 4HR (B, C) showed denser signals than the other groups. However, the group having 3% 4HR (C) showed more localized signal than the group having 1% 4HR (B).

Autogenous implants have been the gold standard of tissue engineering. However, the dimensional stability of autogenous implants for soft tissue reconstruction is very low. Though reports vary, the general resorption rate of free fat graft is 50%[27,28]. The dermis is primarily composed of collagen fiber. Collagen fiber without intentional cross-linking procedure degrades rapidly when implanted into the body[29]. Silk fibroin is slowly biodegradable[18,19]. The complete degradation of natural silk takes approximately two years[30]. Therefore, silk implant does not need cross-linking using chemicals. In this study, the ST3 group developed high levels of connective tissue and vascular channel regeneration without an inflammatory reaction at four weeks postoperatively. The limitation of this study was that long-term observation was not possible. To clarify

long-term dimensional stability, subsequent studies should be of longer duration.

In this study, 4HR was used for silk implants. 4HR is used as an ingredient of oral gargling solution[31], and has antiseptic[21] and analgesic effects[32]. The 4HR combination implant was initially of interest because of its antiseptic effect[24]. The 4HR combination graft formed less foreign body giant cells and developed a higher vascular network (Fig. 5, 6). 4HR can inhibit the formation of foreign body giant cell via diacylglycerol kinase pathway[25]. 4HR also increases vascular endothelial growth factor expression in various cell types (data not shown). However, the effect of 4HR is dose-dependent[23]. ST3 group showed most prominent connective tissue and vascular regeneration at four weeks postoperatively (Fig. 5).

4HR inhibited antibody affinity to antigen (Fig. 1). In a previous study, 5 μM to 50 μM of 4HR has a pronounced inhibition of antigen-antibody complex formation[26]. In this study, much lower concentrations of 4HR also possessed the inhibition effect. As 4HR has long alkyl group and is hydrophobic, the alkyl group may interact with the hydrophobic domain of antibody. Hydroxyl group of 4HR would extrude toward water. These interactions might change the conformation of antibody and influence on its affinity to antigen. ST3 and ST6 groups had lower numbers of lymphocytes. Lymphocytes infiltration is a sign of chronic inflammation, mediated by antigen-antibody reaction. Low level of lymphocytes infiltration in ST3 and ST6 group might be due to the inhibition of antibody reaction by 4HR. Interestingly, the ST3 group showed significantly less giant cells than the ST6 group ($P=0.001$). High concentration of 4HR does not inhibit antibody affinity to antigen[26]. Therefore, the inhibition of giant cell formation by 4HR might be highly concentration dependent. Giant cell formation in the subcutaneous pocket model is transient in the silk biomaterial[33]. This is different from a bone defect model of silk fibroin graft[18,19]. In our study, all groups showed very low level of inflammatory reaction at six weeks postoperatively (data not shown).

Conclusion

In conclusion, 4HR combined silk implants showed high levels of vascular and connective tissue regeneration. However, long-term dimensional stability was not part of this study. Long-term studies should follow.

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