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RESEARCH PAPER

Wound Healing Effect of Collagen-hyaluronic Acid Implanted in Partially Injured Anterior Cruciate Ligament of Dog

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Abstract The purpose of this study was to evaluate the cell compatibility of collagen-hyaluronan (HA) substrate *in vitro* by assessing ACL cell cultures. Additionally, the use of collagen-HA substrate as a dressing material for partial ACL defects as well as its effect on collagen synthesis and angiogenesis were evaluated *in vivo*. The initial attachment and proliferation of dog ACL cells on silk matrix covered with collagen-HA substrate (SMCH) was greater than that observed on silk matrix alone. Silk matrix and SMCHs were implanted as dressing materials into partial ACL defects located at the knees of dogs, and they were harvested six weeks after implantation. A histological evaluation of the collagen-HA substrates revealed the presence of monocytes and the absence of giant cells in all cases. MT staining of the SMCH-grafted group showed a higher level of granulation tissue formation consisting of fibroblasts and collagen fibers compared to the silk matrix-grafted group. In addition, CD31 staining revealed that the SMCH-grafted area showed more blood vessel formation than the silk matrix-grafted area. These results suggest that the collagen-HA substrate was cell-compatible *in vitro* and enhanced collagen synthesis and new blood vessel formation *in vivo*.

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1. Introduction

The human anterior cruciate ligament (ACL) fails to heal after complete rupture, causing knee instability, meniscal damage, and osteoarthritis. Ruptured ACLs are usually surgically replaced, sometimes resulting in less than satisfying outcomes. Patellar tendon autografts and allografts are widely used but are not well suited for ACL reconstruction [1]. The problems associated with autografting include lengthy rehabilitation as well as persistent patellar pain. Likewise, allografts carry the risk of disease transmission, and their procurement is difficult and expensive [2,3].

To overcome the problems associated with autografts and allografts, a synthetic ligament was developed and implanted into patients with ligament injuries. However, healing inside the synthetic ACL was poorly organized, incomplete and unpredictable, as the extent of collagenous infiltration into the textile structure did not increase with the duration of implantation [4]. Despite numerous studies, there is still no biological graft or permanent prosthesis available that is ideally suited for ACL reconstruction.

Various types of biomaterials for ligament and tendon repair have been developed and utilized. However, certain biological responses to these materials have raised questions regarding their biocompatibility. The biocompatibility of implant materials is of major concern in the field of orthopedic surgery [5-8]. For example, host-material interactions occurring at the site of implantation can cause adverse inflammatory reactions after implantation. All biomaterials derived from non-autologous sources cause some type of foreign body response after implantation *in vivo*.

Tissue regeneration using tissue engineering techniques maintains the function and action of the defected tissue while also repairing the original tissue before its defect point. In addition, synthetic non-biodegradable ligaments play a role in mechanical function for short periods of time, but eventually they rupture.

A new tissue engineering strategy was recently attempted for ACL reconstruction. The ideal ACL replacement scaffold should be biodegradable, porous, and biocompatible, show adequate mechanical strength, and be able to promote the formation of ligament tissue and blood vessels. Several groups have reported the potential construction of an ACL scaffold using a carbon copolymer, collagen, silk and biodegradable polymers, such as polyglycolic acid (PGA) and polylactic acid (PLA) [9-15].

Until recently, biodegradable ligaments have lacked satisfactory biocompatibility and physical properties. However, recent research has presented silk materials with satisfactory biocompatibility and physical properties [16]. Silk is obtained from the silkworm *Bombyx mori*, and has been used as a biomedical suture material for centuries. Silk contains 75% (w/w) fibroin proteins (with light chains 25 kDa and heavy chains 325 kDa in size) and 25% (w/w) sericin. A degradation half-life of 6 weeks was reported after subcutaneous implantation of silk in rat; specifically, the silk fibers lost tensile strength within 1 year *in vivo* and were completely degraded within 2 years.

Pioneering research on the reconstruction of tendons and ligaments using silk has been performed by Kaplan and Altman [5]. These authors were able to replace natural ligaments with artificial constructs showing mechanical properties similar to those of natural ligaments by twisting silk threads. However, this method is limited due to its low levels of cell adhesion and growth. Hence, a RGD sequence (arginine-glycine-aspartic acid) was introduced into the replacement material, resulting in improved adhesion and growth of stem cells and ligament fibroblasts [7,15].

In this study, a novel type of bioartificial ligament was designed by embedding parallel silk fibers within a lyophilized collagen-HA acid substrate. To evaluate the effect of collagen and HA on ACL regeneration, ACL growth was investigated *in vitro*, and collagen synthesis and angiogenesis were evaluated during partial ligament defect *in vivo*.

2. Materials and Methods

2.1. Preparation of silk matrix and silk matrix covered with collagen and hyaluronic acid (SMCH)

Silk suture was purchased from Won Corporation (Korea), and the silk matrix was woven on a loom. The silk matrix was then processed by removing the sericin, which is a glue-like protein that coats native silk fibrin, using an aqueous solution containing 0.02 M Na_2CO_3 and 0.3% Ivory detergent [17].

The silk matrix was then soaked in an ECM mixed solution (collagen, hyaluronic acid (HA) and chodroitin-6-sulfate (CS)) and lyophilized. Type I atelocollagen powder (Bioland, Korea) was dissolved in 0.001 M HCl at a concentration of 10 mg/mL, after which HA (sodium hyaluronate Phama grad 200, Novamatrix, Kibun Food Chemi FA Co., Japan) was dissolved at a concentration of 10 mg/mL in 0.05 M acetic acid and CS (Sigma Chemical Company, St. Louis, MO) was dissolved at a concentration of 1 mg/mL in HA solution. Collagen-HA substrate was composed of 2 parts collagen solution and 1 part HA-CS solution. Silk scaffold and collagen-HA-CS mixing solution were then added to the mold, which was then lyophilized by freeze-drying (Samwon Freezing Engineering Co., Korea) at −80°C for 48 h in order to obtain a composite silk scaffold.

For cross-linking, the SMCH was incubated in 20 mL of 40% (v/v) ethanol containing 50 mM 2-morpholineoethane sulfonic acid (MES, Fluka Chemic AG) (pH 5.5) for 30 min at room temperature. The composite silk scaffold was then immersed in 20 mL of 40% (v/v) ethanol containing 50 mM MES (pH 5.5), 24 mM 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (Fluka Chemic AG), and 5 mM *N*-hydroxysuccinimide (Fluka Chemic AG) for 12 h at room temperature. Once the reaction was complete, the composite silk scaffold was washed twice in 0.1 M Na_2HPO_4 (pH 9.0) for 12 h. The scaffolds were then washed twice in 1 M NaCl for 6 h, then in 2 M NaCl for 2 days, and finally rinsed with distilled water [18,19]. The scaffolds were lyophilized by freeze-drying and sterilized with γ-irradiation at 10 KGy.

2.2. Dog ACL cell culture

ACL tissue was obtained from dogs while ACL fibroblasts (ACLs) were obtained using an enzyme digestion method. ACL tissue was harvested from dogs undergoing total ACL reconstruction and transferred to the laboratory in Dulbecco's modified Eagle medium (DMEM). All of the synovial tissue was then cleaned and cut into 1 mm^3 slices. ACLs were isolated from the chopped tissue using 2 mg/mL of type I collagenase (Sigma Chemical, U.S.A.) for 60 min at 37°C, then cultured routinely in DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator.

Sterilized (γ-irradiation) silk matrix and SMCHs were placed in a 12 well-plate. The scaffolds were inoculated with 500 μL of the cell suspension at a concentration of 5×10^4 cells/well by direct pipetting, incubated for 1 h at

37°C/5% CO_2, and then added to 1 mL of DMEM. After seeding, the silk matrix and SMCHs were cultured in the appropriate amount of DMEM (10% FBS) for 18 days for the MTT assay (n = 4).

2.3. Proliferation assay of ACL cells

Cell proliferation was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma) assay. For the MTT assay, cell-cultured scaffolds were transferred to a 12-well plate, and serum-free DMEM was added to each well supplemented with MTT (0.33 mg/mL) (n = 4). Isopropyl alcohol (1 mL) containing 0.04 N HCl was then added, and the 12-well plate was shaken slowly for 15 min. The absorption was measured at 540 nm.

2.4. Statistical analysis

The data for ACL growth was evaluated statistically using student's t-test. The data are represented as the mean ± SD. $p < 0.05$ were considered significant.

2.5. Animal experiments

The Animal Welfare Committee of the Catholic University of Korea approved the experimental protocol. Four dogs (beagles, weighing an average 12 kg) were used in the present study. They were fed common dog food throughout the experimental period. The dogs used in the experiments were adult dogs without any special history.

The experimental dogs were anesthetized by intramuscular injection of 50 ~ 75 mg/kg of ketamine (Yuhan Corp., Seoul, Korea) and 20 mg/kg of rompun (Bayer Korea Ltd., Korea). The ACL was exposed by making a sterile incision in the skin along the midline of the knee joint on both sides using the medial parapatellar approach (Fig. 3).

Using a scalpel, the midpoint of the substance on the inside of ACL was incised 2 mm in length and for half of its entire thickness. After careful hemostasis, the incised area of the left cruciate ligament was wrapped with the silk matrix and sutured with Dexon (absorbable suture material). The right knee joint was wrapped with SMCH that had been treated with collagen and HA.

The dogs were sacrificed and examined 6 weeks after implantation, and the ACL was obtained from both sides. HE and MT staining as well as immunochemistry were performed to assess cell migration and angiogenesis in the implanted silk matrix and SMCH.

Masson's trichrome (MT) staining was performed on 4 ~ 5 μm thick serial sections mounted on poly L-lysine-coated slides. The sections were deparaffinized, and mordanted in Bouin's solution, microwaved for 1 min and then allowed to stand for 15 min. After washing with distilled water for 5 min, the sections were treated with Weigert's working hematoxylin for 10 min, rinsed in distilled water for 5 min, soaked in Biebrich scarlet solution and then treated with phosphotungstic/phosphomolybdic acid. The sections were then transferred and maintained in aniline blue solution for 5 min. Finally, washing was performed with 1% acetic acid for 1 min followed by distilled water.

CD31 immunohistochemical staining was performed as follows. The sections were deparaffinized and rehydrated. The endogenous peroxidase activity was blocked using 3% H_2O_2, and the nonspecific reaction of bovine serum albumin and non-immune serum was performed with anti-CD31 monoclonal antibody (Dako, Carpinteria CA) followed by standard immunohistochemistry. A Dako Envision kit was used as the secondary antibody while amino ethyl carbazole (AEC) was used as the substrate. The slides were counterstained with Mayer's hematoxylin, rinsed in tap water and then mounted.

The foreign-body reaction was characterized on a 3 level scale 6 weeks post-implantation [5]. The relative level was scored on the light microscopy image as follows: mild reaction included the presence of histiocytes and foreign-body giant cells (lower than 200 of immune cells), moderate reaction indicated histiocytes and foreign-body giant cells as well as clusters of lymphocytes and a few plasmacytes and extensive reaction included histiocytes, foreign-body giant cells and diffuse, scattered lymphocytes and plasmacytes (higher than 1,000 of immune cells).

3. Results and Discussion

3.1. Proliferation of ACL cells on matrix *in vitro*

Fig. 1 shows the silk matrix (A) and silk matrix covered with collagen and HA (SMCH) (B). The silk matrix weaved by the loom had a twisted structure, whereas the SMCH, which was covered with collagen-HA substrate, had a porous sponge structure.

Cell proliferation dependent on time was observed during the 18 days of culture, as determined by MTT analysis (Fig. 2). The initial seeding cell number was the same (5×10^4 cells) in each group. After two days of culture, the cell number was determined to be 2.82×10^4 cells for the silk matrix group and 3.8×10^4 cells for the SMCH group. After 18 days of culture, the cell number was estimated to be 8×10^4 cells for the silk matrix group and 14.5×10^4

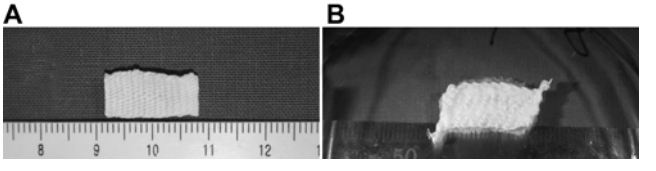

Fig. 1. Gross appearance of silk matrix: (A) silk matrix and (B) silk matrix covered with collagen and hyaluronic acid (SMCH).

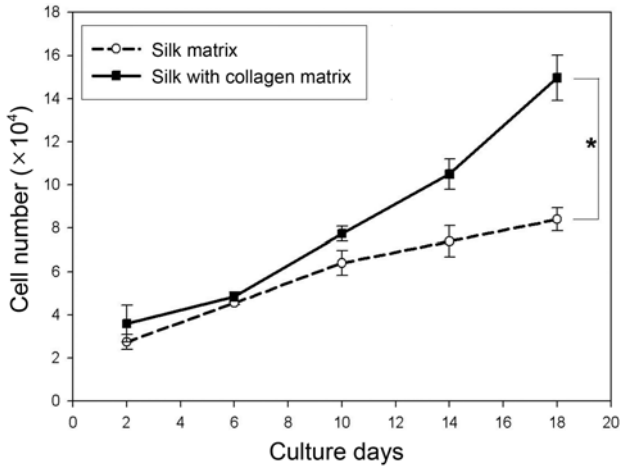

Fig. 2. Measurement of dog ACL cell growth on silk matrix, and silk matrix covered with collagen and hyaluronic acid (SMCH) *in vitro* by MTT assay. (*$p < 0.05$, n = 4 per group).

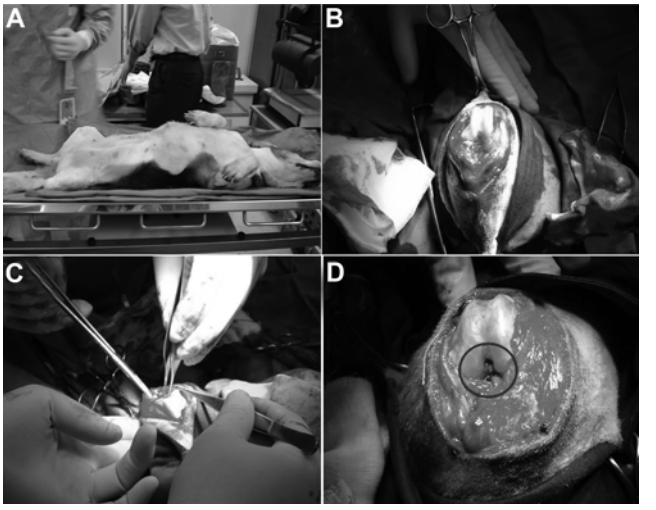

Fig. 3. Procedure for implantation of silk matrix and silk matrix covered with collagen and hyaluronic acid (SMCH) in the knee joint of dog. The dog was anesthetized (A) and its ACL exposed by making a sterile incision in the skin along the midline of the knee joint using the medial parapatellar approach (B). Using a scalpel, the midpoint of the substance on the inside of ACL was incised 2 mm in length and for half of its entire thickness (C). Then the incised area of the left cruciate ligament was wrapped with silk matrix and sutured with Dexon while the right knee joint was wrapped with SMCH that had been treated with collagen and hyaluronic acid (D).

cells for the SMCH group. The cell density on the SMCH was increased significantly by an average 80% compared to the silk matrix (Fig. 2).

In this study, collagen-HA substrates played an important role in increasing cell attachment and proliferation. Collagen is the most abundant protein within the mammalian extracellular matrix (ECM) and can be easily isolated from animal tissues or organs. Further, it has been widely applied in tissue engineering since it interacts with cells in connective tissues and emits essential signals for regulation of cell anchorage, migration, proliferation, and differentiation. Collagen shows excellent biocompatibility and safety as a result of its intrinsic biological characteristics such as biodegradability and weak antigenicity.

As with collagen, HA, which is clinically available, has viscoelastic and lubricating properties that allow its wide application in ophthalmologic and joint regions. During skin development in the embryo, HA is expressed to a high extent, whereas it is involved in cell migration and differentiation during wound healing [20]. Addition of HA to collagen gel induces rapid fibroblast proliferation and increases the levels of tubulin and actin in the cytoskeleton [21,22]. Furthermore, when cultured on a HA/collagen sponge, fibroblasts proliferate and produce more collagen compared to culture on a simple collagen sponge. Specifically, fibroblasts cultured on collagen sponges containing 5, 10, or 20% (all w/w) HA were increased in cell proliferation [23].

The MTT assay results suggest that the ACL cells grew poorly and did not attach well when using silk matrix alone. The SMCH designed in the present study had identical mechanical properties as that of a silk matrix and increased the adhesion and proliferation of cells by lyophilized collagen-HA substrates.

3.2. Histological analysis after grafting *In vivo*

Six weeks after the operation, there were no signs of a rupture of the repaired ACL in the dogs. Cross-section of slides was performed in the center of the ACL defects, and each of the three slides was observed directly.

In the SMCH group, vascularity and collagen-like tissue was observed (Fig. 4). There was no evidence of angiogenesis or granulation tissue formation (Fig. 4A). Mild inflammatory responses were observed in the silk matrix, whereas monocytes and blood vessel formation were observed in the SMCH group (Figs. 4A and 4B). The inflammatory reaction of the matrix was observed histologically (Figs. 4C and 4D). Heavy infiltration of fibroblast-like cells along with new collagen formation was observed in the SMCH group, and no foreign-body reaction was induced (Figs. 4C and 4D).

In this area of the cross-section, MT staining showed collagen fibers whose purpose was regeneration of the ACL defect. In the silk matrix-grafted group, collagen-like tissue was observed in about 20% of the defect area (Fig. 5A). However, in the SMCH-grafted group, collagen fibers were observed mostly on the wound healing area compared to that of the silk matrix-grafted group in the cross-section.

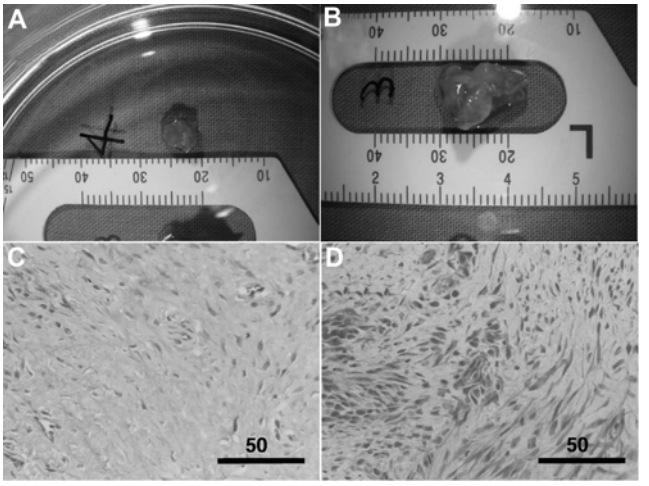

Fig. 4. Histological comparison of the silk scaffold (A and C) and SMCH (B and D). Cross-section was performed in the center of defected area. Mild reactions were observed in the silk matrix group and SMCH group. H&E stain: (C, D) = × 400, Scale bar = 50 μm.

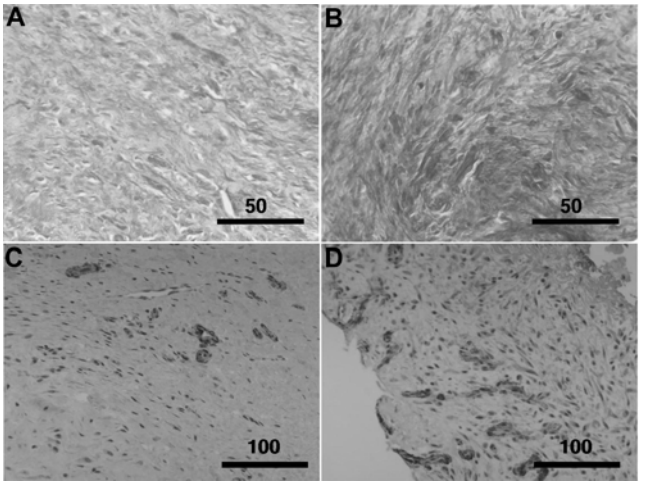

Fig. 5. Histopathological comparison of the silk matrix (A and C) scaffold area and the SMCH area show new granulation tissue (B) and blood vessel formation (D) in the SMCH grafts at the partially defected area. (A and B: Trichrome stain, C and D: CD31 staining). Original magnification: (A, B) × 400; (C, D) × 200, Scale bar = (A, B) 50 μm; (C, D) 100 μm.

Collagen and hyaluronan play several cellular functions by promoting the adhesion, migration, proliferation and secretion of ECM components and cytokines. When a collagen sponge soaked in 0.3% (v/v) HA solution was transplanted onto wounds, cell migration and collagen production were accelerated, in contrast to controls receiving only a simple collagen sponge [24-27].

Immunohistological observation of CD31 staining revealed the formation of new blood vessels (Figs. 5C and 5D). In the SMCH-grafted area (Fig. 5D), new blood vessels were observed more than in the silk matrix-grafted area (Fig. 5C).

Many investigators in previous studies have tried to increase the rate of blood vessel formation for the repair or reconstruction of damaged ligaments and tendons. Yoshikawa *et al*. reported an increase in VEGF expression, fibroblast proliferation, and angiogenesis in a patellar tendon graft during its early phase following ACL reconstruction in a rabbit model [28]. The grafted tendon showed signs of necrosis when there was no vascular contribution within three weeks after the transplantation. These findings indicate that angiogenesis is a very important factor in ACL repair. While the ACL shows very limited healing capabilities, the medial collateral ligament (MCL) has a relatively good healing capacity, and it responds to injury in a manner similar to that of other vascular soft tissues. Bray *et al*. reported that the superior capacity of the MCL to increase its blood supply through angiogenesis and increased blood flow is essential for ligament healing, and this may be the major difference in healing potential between the ACL and MCL [29]. Several research studies have demonstrated that neovascularization is essential for ligament healing and regeneration during the early phase [30-36].

Kobayashi *et al*. found that enhanced neovascularization and formation of granulation tissue induces the healing of a partially injured ACL with growth factor [37]. A sheep study by Yoshikawa *et al*. using a VEGF solution demonstrated that newly formed vessels and infiltrative fibroblasts are more abundant in tendons that have been soaked in VEGF solution prior to grafting into a ruptured ACL, compared to tendons that have been soaked in PBS [38]. Unfortunately, growth factor is very expensive.

High molecular weight hyaluronan in the ECM has been shown to inhibit angiogenesis [39]. In contrast, low molecular weight hyaluronan oligosaccharides have been shown to promote angiogenesis in several studies [40-42]. Halici *et al*. reported that injected sodium hyaluronate increased VEGF and type VI collagen expression in a defected achilles-tendon in rabbit, suggesting that sodium hyaluronate may be partly involved in the regulation of angiogenesis [43].

4. Conclusion

A new biomaterial with strong mechanical properties and slow degradation is currently needed for the orthopedic surgery. A number of investigators have recently demonstrated the possibility of ligament reconstruction using silk [24]. Silk matrix can be very convenient for repairing connective tissue in orthopedic patients, and this study along with previous others suggest that ACL reconstruc-

tion using silk matrix is feasible. However, the silk matrix system alone is not a suitable material for ACL reconstruction.

Therefore, in this study, we compared the effect of collagen-HA substrate on angiogenesis, cell migration, and collagen synthesis during the initial phase of ACL regeneration in a canine model. The collagen-hyaluronan substrates increases the rates of cell migration compared to that of silk scaffold. Moreover, these substrates induced angiogenesis and collagen synthesis, which is essential for the initial repair of damaged ligaments.

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