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ORIGINAL ARTICLE

The Application of Fibrin/Hyaluronic Acid–Poly(L-Lactic-*co*-Glycolic Acid) Construct in Augmentation Rhinoplasty

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Abstract Although many graft materials have been used for augmentation rhinoplasty, an ideal graft has not yet been developed. As the field of tissue engineering has been developing, it has been applied to the reconstruction of many organs, but its application in the rhinoplasty field is still limited. This study evaluated the utility of allogenic chondrocytes with fibrin/hyaluronic acid (HA)–poly(L-lactic-*co*-glycolic acid) (PLGA) constructs in augmentation rhinoplasty. Chondrocytes from rabbit auricular cartilage were isolated and cultured with fibrin/HA hydrogels and implanted into PLGA scaffolds. After 8 weeks of *in vitro* culture, the scaffolds were implanted in the nasal dorsum of six rabbits. Eight weeks postoperatively, the implanted sites were evaluated with gross, radiologic, and histologic analysis. *In vitro*, more than 90% of the seeded chondrocytes in the PLGA scaffolds survived for 2 weeks, and they produced a large amount of extracellular matrix and were well differentiated. The grafts maintained their initial shape for 8 weeks after implantation. Radiological and histological evaluations showed that the structure was well maintained with minimal inflammatory response and appropriate elevation levels. However, the formation of neo-chondrocytes was not observed. PLGA scaffolds seeded with fibrin/HA and allogenic chondrocytes can be a biocompatible augmentation material in rhinoplasty in the future.

Keywords Rhinoplasty · Augmentation materials · Poly(L-lactic-*co*-glycolic acid) · Chondrocyte · Tissue engineering

1 Introduction

Augmentation rhinoplasty, which is a technique used to elevate the nasal dorsum using various materials, is one of the most commonly performed rhinoplasty procedures among Asians, including Koreans [1]. In the practice of augmentation rhinoplasty, various materials such as autologous cartilage, autologous fat, silicone or Gore-Tex® are implanted to elevate the nasal dorsum. Therefore the selection of appropriate materials is one of the most important factors for successful rhinoplasty. The implantation materials used for augmentation rhinoplasty are largely classified into autologous materials and artificial materials. The use of autologous grafts is the best option in terms of biocompatibility and stability. However, additional incisions and invasive surgical procedures are needed to obtain autologous grafts, and there is a limit in obtaining a sufficient volume of graft material. On the other hand, artificial grafts can be appropriately designed to acquire sufficient quantities of materials, resulting in excellent cosmetic outcomes. However, they have the disadvantage of being difficult to maintain permanently and can lead to rejection of the graft [2–4].

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With the evolution of tissue engineering, it has become possible to reconstruct tissues *in vitro*. Thus, tissue engineering can be applied to maintain and restore normal anatomical structures. For example, regenerated cartilage or bone tissue through tissue engineering has been used for the reconstruction of joints, auricle, maxilla, and trachea [5–11]. However, applications in the field of rhinoplasty are still limited. For cartilage regeneration through tissue engineering, a scaffold for regeneration with autologous cells is needed. Nasal septal cartilage can be easily obtained in the course of rhinoplasty. Therefore, a suitable scaffold is essential for the regeneration of implantation materials for augmentation rhinoplasty [12]. The requirements of the scaffold for rhinoplasty are as follows. First, it should be a three-dimensional structure that can be made into a desired shape. Second, environmental conditions for cell proliferation and differentiation should be made possible. Finally, there is a need for an appropriate microenvironmental element to prevent cell loss [13–15].

Poly(L-lactic-*co*-glycolic acid) (PLGA) is the most widely used biodegradable polymer in the field of regenerative medicine because of its adjustable degradation, mechanical strength, suitable processability, and biocompatibility [16, 17]. Fibrin/hyaluronic acid (HA) gels are known to be useful for culturing allogenic chondrocytes, but there is a limitation in that their mechanical strength is reduced by rapid degradation *in vivo* [18]. Therefore, in this study, we aimed to regenerate cartilage tissue by implanting PLGA scaffolds with allogenic chondrocytes cultured within a fibrin/HA gel, and we investigated the usefulness of this system as an implantation material.

2 Materials and Methods

2.1 Isolation of chondrocytes

Chondrocytes were isolated from cartilage tissue of the auricle of 12-week-old New Zealand white rabbits. Auricular cartilage was harvested under anesthesia using tiletamine (4.0 mg/kg; Virbac Ltd., Carros, France) and zolazepam (4.0 mg/kg; Virbac) in aseptic conditions. The cartilage was washed with phosphate-buffered saline (PBS) after being finely minced, and collagenase (Worthington Biochemical, Lakewood, NY, USA) was added and stored at 37 °C for 5 h to dissolve cartilage tissue. The cells were filtered using a 100-μm nylon cell strainer (Falcon, Franklin Lake, NJ, USA) and centrifuged at 1700 rpm for 10 min. After washing twice with PBS, cell pellets were cultured in Dulbecco's Modified Eagle medium (DMEM, Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco BRL), 100 U/mL penicillin G (Gibco BRL), and 100 μg/mL streptomycin (Gibco BRL). Cells were plated at a density of 1.5×10^5 cells/cm^2 and incubated in an environment of 37 °C and 5% CO_2. The culture medium was changed every day and the cells were cultured in two passages before being used in the experiment.

2.2 Preparation of fibrin/HA composite gel

The cultured chondrocytes were pelleted by centrifugation and suspended in a solution containing fibrinogen (9–18 mg/mL; Mokam Research Center, Suwon, South Korea) and HA (molecular weight 3000 kDa; 10 mg/mL; LGCI, Daejeon, South Korea). Fibrinogen and HA were mixed at a volume ratio of 10:1. Afterwards, a chondrocyte suspension at 1×10^6 cells/mL was homogeneously mixed with 110 KIU/mL aprotinin (Mokam Research Center), 60 U/mL thrombin (1000 U/mg protein; Sigma, St. Louis, MO, USA), fibrin stabilizing factor XIII, and 50 mM $CaCl_2$. The formed fibrin/HA mixture (250 mL) was dispensed into an empty petri dish to form a gel. It was then transferred to 6-well plates and cultured in DMEM supplemented with 10% FBS and antibiotics.

2.3 Preparation of PLGA scaffold

Cylindrical PLGA scaffolds (Regen Biotech, Sung-nam, Korea) with a pore size of 250–400 μm, a length of 50 mm, an inner diameter of 30 mm, and a thickness of 3 mm were designed as a curved patch-type graft having a width of 10 mm and a height of 5 mm. The ratio between lactic acid and glycolic acid was 70:30 and the molecular weight was 80–145 kDa. The graft was completely immersed in 75% ethanol solution under aseptic conditions for hydration and stored in a refrigerated state (4 °C) overnight. Ethanol was completely removed by repeated washing with sterile pyrogen-free water, PBS, and serum-free medium. Sterilization was carried out with ethylene oxide gas using an EOG-300 apparatus (Delta Medical, Ansan, Korea). A mixture of cultured chondrocytes (1×10^6 cells/mL) and fibrin/HA gel was implanted into a sterile PLGA scaffold. The scaffold in which chondrocytes were transplanted was cultured in chondrogenesis-defined media [DMEM with 1.0 mg/mL insulin from bovine pancreas, 0.55 mg/mL human transferrin, 0.5 mg/mL sodium selenite, 50 mg/mL ascorbic acid, 100 mM dexamethasone, 40 mg/mL L-proline, 1.25 mg/mL bovine serum albumin, and 100 mg/mL sodium pyruvate (Sigma-Aldrich, St. Louis, MO)] for 8 weeks before implantation (Fig. 1A).

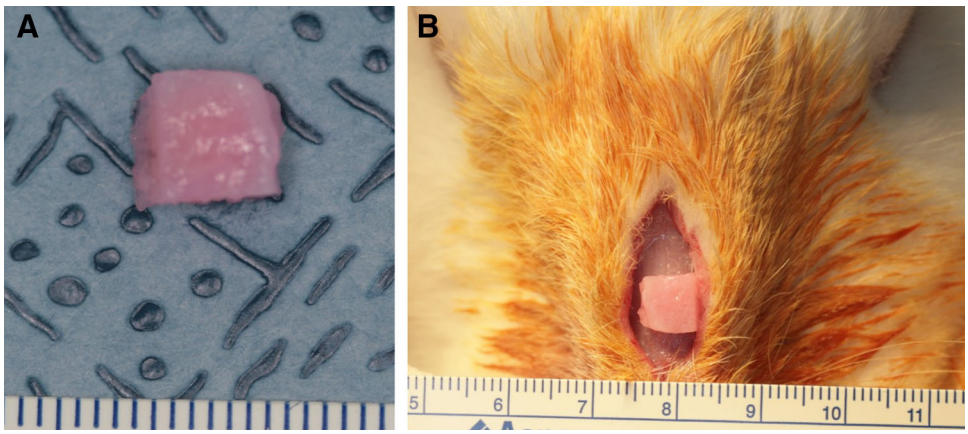

Fig. 1 Preparation and implantation of fibrin/HA–PLGA scaffold. **A** Fibrin/HA gel and chondrocyte mixture was implanted in the designed scaffold. The width and height were 10 mm × 5 mm and the thickness was 3 mm. **B** The prepared construct was inserted on the dissected supraperiosteum of the nasal dorsum

2.4 Scanning electron microscopy (SEM)

PLGA scaffolds seeded with chondrocytes were observed using SEM to evaluate the pore connectivity and the degree of cell implantation in the pore spaces. The scaffolds were processed in the form of a hexahedron ($5 \times 3 \times 1$ mm^3) and fixed to the sample holder. After platinum coating using an SC500 K plasma sputter (Emscope, West Sussex, UK), each sample was evaluated using an S-4800 SEM (Hitachi, Tokyo, Japan) at 10 or 15 kV.

2.5 Assessment of *in vitro* chondrocyte survival: calcein acetomethoxy/ethidium homodimer-1 assay

The viability of chondrocytes seeded into the PLGA scaffolds was assessed using a LIVE/DEAD Viability/Cytotoxicity Kit with calcein acetomethoxy (calcein-AM) and ethidium homodimer-1 (EthD-1) (Invitrogen, Carlsbad, CA, USA) at 2 weeks [19]. Briefly, the chondrocytes seeded into PLGA were incubated in 2 μM calcein-AM and 1 μM EthD-1 for 30 min at 37 °C. After mounting on a slide containing FluorSave (345789 EMD Biosciences, Gibbstown, NJ, USA), fluorescence images were acquired using a Microphot FXA digital fluorescence microscope (Nikon, Melville, NY, USA). Cell viability was quantified by dividing the number of live cells (green) by the total number of cells (green + red) in five randomly selected slides. Measurements were quantified using ImageJ software (National Institutes of Health, USA) and expressed as a percentage.

2.6 *In vitro* histologic assessment

Fibrin/HA gel and chondrocytes were transplanted into PLGA scaffolds and cultured *in vitro*. Histological evaluation was performed at 2, 4, and 8 weeks. After the incubation period, the cells were fixed with 10% neutral-buffered formalin for 24 h. The tissue was formed into a paraffin block and cut to a thickness of 4 μm. After staining with hematoxylin–eosin (H&E) and safranin-O (Sigma Aldrich, St. Louis, Mo., USA), samples were observed by light microscopy.

2.7 Animal model and surgical techniques

Animal care and procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and all experiments were approved by the Committee for Ethics in Animal Experiments of the Ajou University School of Medicine. Six male New Zealand white rabbits (Samtaco, Osan, Korea) weighing 2.5–3.0 kg at 12 weeks of age were stabilized in the central animal laboratory for more than a week. Animals were kept in a single cage at 21 ± 1 °C, allowing food and water to be freely accessed. Lighting was maintained from 8:00 am to 8:00 pm. The rabbits were anesthetized by the same method as described above and fixed on prone position. Vertical incision was applied to the skin of the nasal dorsum using a No. 15 blade. After exposing the nasal bone and upper lateral cartilage, the prepared PLGA scaffold was placed on the supraperiosteum of the dorsum (Fig. 1B). Subsequently, the skin and subcutaneous tissue were tightly sutured.

2.8 Assessment of gross appearance

After 8 weeks of implantation, the nasal dorsum was depilated and the overall shape of the implant site was assessed. The degree of dorsal augmentation, the status of skin incision and the presence of postoperative complications were evaluated by two independent examiners.

2.9 Radiologic assessment

Computed tomography (Brilliance 64, Philips, Eindhoven, Netherlands) was performed under anesthesia at 8 weeks after implantation. Axial and coronal images were taken in 1-mm increments.

2.10 *In vivo* histologic assessment

Animals were sacrificed after the end of the experiment. The nasal dorsum including bone, upper lateral cartilage, nasal septum, and graft site was excised and prepared for light microscopy. After fixing for 24 h using 10% neutral-buffered formalin, the tissue was embedded in paraffin and sectioned at a thickness of 4 μm. After staining with H&E and safranin-O, the specimens were examined under a light microscopy.

3 Results

3.1 Identification of chondrocytes seeded in the pores of PLGA scaffolds

The SEM images of the PLGA scaffolds confirmed that they have good porosity with sufficient space for interconnection between the pores (Fig. 2A). After implantation of the allogenic chondrocytes from the fibrin/HA gel into the PLGA scaffold, SEM images were acquired again. Allogeneic chondrocytes were confirmed to be well seeded to the pores of PLGA scaffolds (Fig. 2B).

3.2 Survival and histologic evaluation of chondrocytes seeded in PLGA scaffolds

The viability of chondrocytes seeded in the PLGA scaffolds was assessed by calcein-AM/EthD-1 assay after 2 weeks (Fig. 3). More than 90% of chondrocytes seeded in the PLGA scaffolds were confirmed to survive.

Histological evaluation after H&E staining showed chondrocyte survival after 2, 4, and 8 weeks of culture (Fig. 4A–C). In particular, after 8 weeks of culture, chondrocytes that formed more prominent lacuna were observed (Fig. 4C). Cartilage formation and extracellular matrix production were confirmed by safranin-O staining, and remarkable development of cartilage tissue was observed in all three periods (Fig. 4D–F). Although no mature forms of chondrocytes were present in all three periods at high magnification, extracellular matrix production was observed after 4 weeks of culture (Fig. 4G–I). The amount of extracellular matrix produced was even higher after 8 weeks of incubation (Fig. 4I).

3.3 Gross, radiologic and histologic evaluations of the implanted PLGA constructs in animal model

The rabbits used in all experiments were healthily maintained until they were sacrificed 8 weeks after implantation. After 8 weeks of implantation, a gross evaluation of the implant site was performed (Fig. 5A, B). There was no sign of postoperative infection or inflammation, and in all experimental groups, the eminence degree of the nasal dorsum was maintained for 8 weeks postoperatively. In

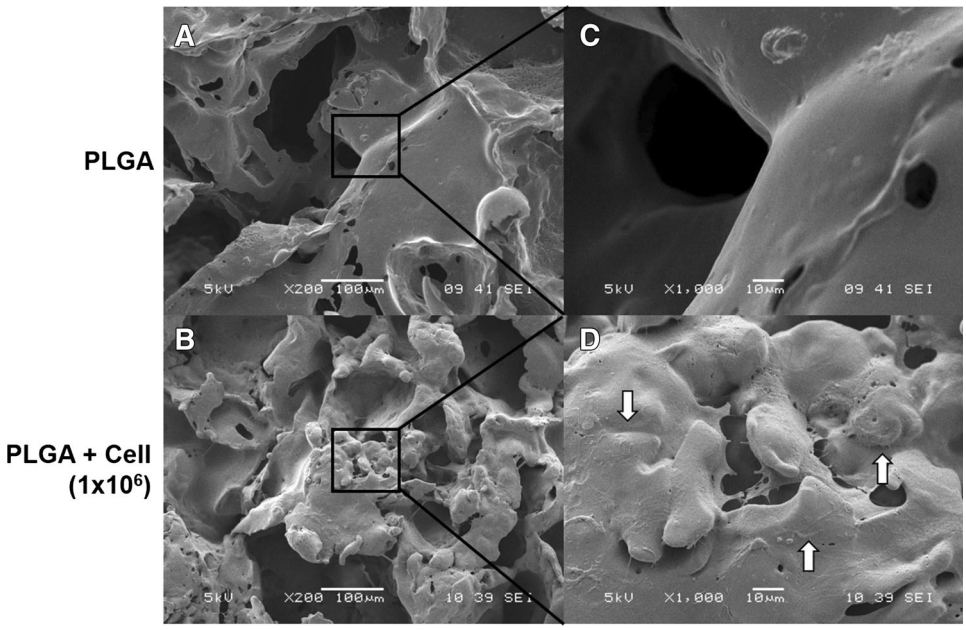

Fig. 2 **A**, **B** Scanning electron microscopy images of cell-seeded PLGA scaffold. **C** Scaffold before cell seeding. Prepared PLGA scaffold showed good porosity and sufficient interconnection between pores. **D** Scaffold after seeding of chondrocytes and fibrin/HA gel mixture. Allogenic chondrocytes containing fibrin/HA gel were settled (white arrow)

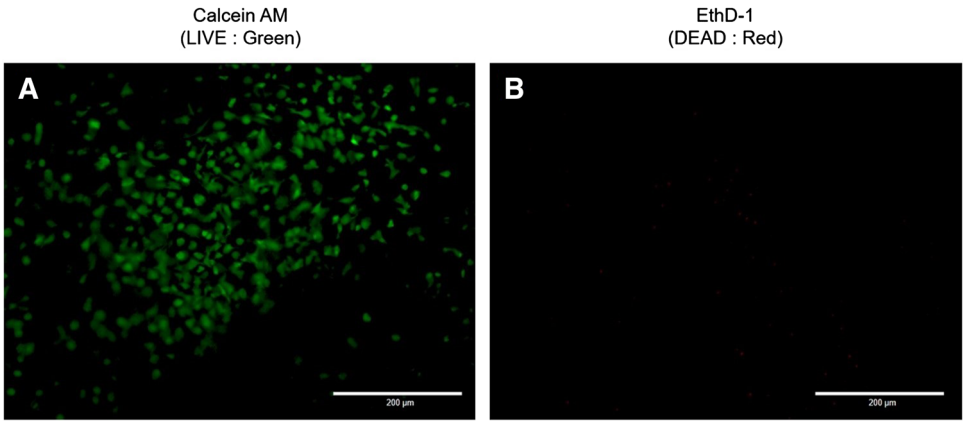

Fig. 3 LIVE/DEAD assay of seeded allogenic chondrocytes on the PLGA scaffold. **A**, **B** Live cells were stained with green-fluorescent calcein-AM and dead cells with red-fluorescent EthD-1. Over 90% of cells survived after the implantation

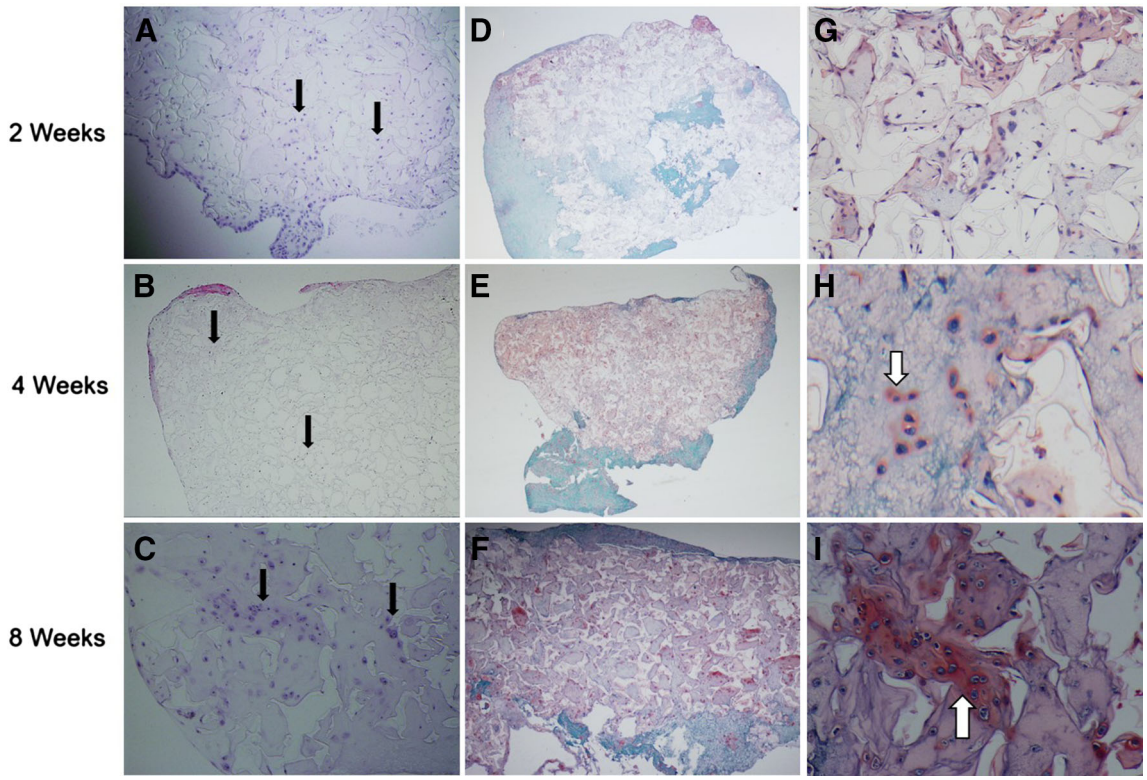

Fig. 4 Histologic evaluation of seeded allogenic chondrocytes on the PLGA scaffold. **A–C** Hematoxylin and eosin (H&E) staining of construct under low magnification (×40). Surviving chondrocytes were observed at all periods of culture (black arrow). **D–F** Safranin-O staining of construct under low magnification (×40). Remarkable development of cartilage tissue was observed in all three periods. **G–I** Safranin-O staining of construct under high magnification (×400). The production of extracellular matrix was found after 4 weeks of culture (white arrow)

radiologic studies using computed tomography in 1-mm increments, the implanted scaffolds were maintained for 8 weeks without radiographic abnormalities. Histologic evaluation was performed after 8 weeks with a complete resection including the implanted scaffold, skin, and soft tissue. Under low magnification, the shape of the scaffold was well maintained without inflammation, and damage to the soft tissue was not observed (Fig. 6A). Safranin-O staining was used to confirm cartilage formation in the implanted scaffold. Significant formation of new cartilage tissue was not observed after 8 weeks, but formation of fibrotic tissue containing viable fibroblasts was confirmed (Fig. 6B).

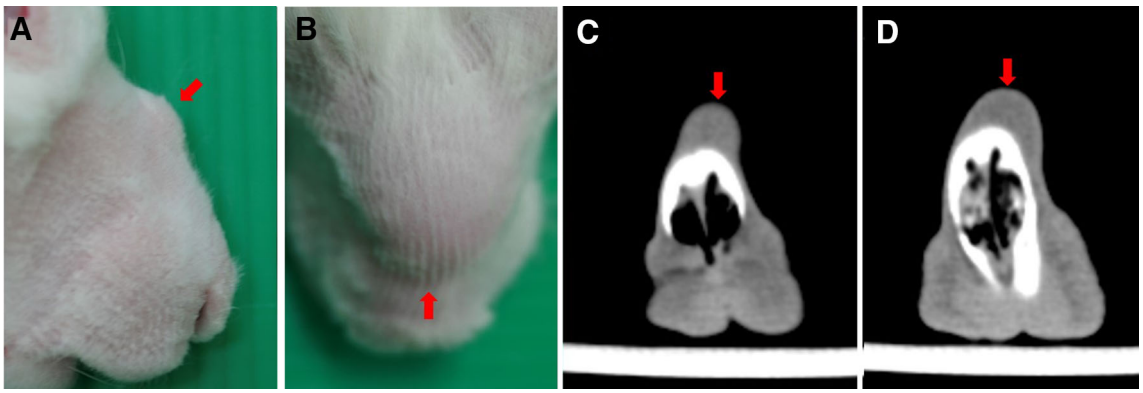

Fig. 5 Gross and radiologic evaluation of implanted fibrin-HA/PLGA scaffold in animal model. **A**, **B** Lateral and frontal photographic view of nasal dorsum after 8 weeks of implantation. **C**, **D** Axial and coronal view of computed tomographic findings of implanted PLGA scaffold construct after 8 weeks of implantation. The implanted scaffold was well maintained without any specific inflammatory reaction (red arrow)

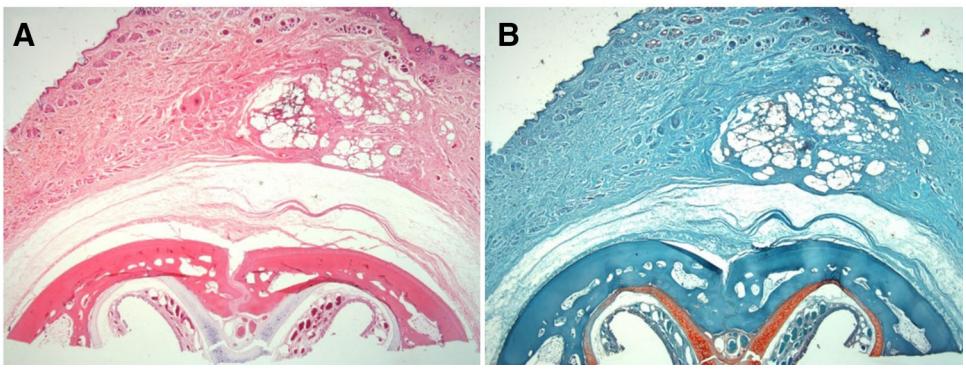

Fig. 6 Histologic evaluation of implanted fibrin/HA–PLGA scaffold in animal model. **A** Hematoxylin and eosin (H&E) staining of implanted construct under low magnification (×40) after 8 weeks of implantation. **B** Safranin-O staining of implanted construct under low magnification (×40) after 8 weeks of implantation. The implanted scaffold was well maintained without inflammation and reduction in volume

4 Discussion

In the past, artificial materials such as silicone and Gore-Tex® have been most commonly used in augmentation rhinoplasty, especially in Asians [4, 20, 21]. However, these artificial grafts have side effects due to immune reaction or infection, thus it is difficult to maintain them permanently. In recent years, many studies have been carried out on new biomaterials for rhinoplasty, but there have been many limitations in their development.

Advances in tissue engineering have facilitated the development of biocompatible and functional materials to repair and replace human tissues and organs. Tissue engineered biocompatible materials can be used for facial cosmetic surgery to reconstruct the aesthetic aspects of facial contours [12]. In other words, *in vitro* cultured autologous cells can be implanted into the nasal dorsum for augmentation rhinoplasty [22, 23]. Yanaga et al. reported successful results of chondrogenesis and dorsal elevation by autologous chondrocytes derived from the conchal cartilage for up to 24 months [22]. In another study, dermal fibroblast cultures with HA suspensions showed successful results after injection into the nose of 11 patients [23]. Although these studies have shown meaningful results, these methods present several limitations. Implantation methods involving the injection of cells and cell-scaffold constructs could not be used to position an implant precisely at the desired location, resulting in unpredictable results. Another recent study reported the formation of cartilage tissue after subcutaneous implantation of autologous nasal chondrocytes and fibrin constructs into nude mice [24].

Fibrin glue possesses abundant fibronectin, which is essential to the matrix of cartilage tissue [18], while HA is a major component of the extracellular matrix and is a biocompatible and biodegradable material that has been used in a variety of medical applications [25–27]. Therefore, the fibrin/HA gel provides a favorable environment for maintaining the characteristic phenotype of chondrocytes and synthesizing the extracellular matrix. However,

the rapid degradation of fibrin/HA gels causes limitations in maintaining proper cartilage formation [18]. Furthermore, as described above, the initial shape of the construct cannot be maintained with a simple injection method. In this study, we applied a rigid scaffold, PLGA, that can be formed into a desired shape before augmentation rhinoplasty and precisely implanted at a desired position.

PLGA is FDA approved and used in a variety of medical applications [28]. PLGA has an appropriate degradation rate and excellent mechanical properties such as toughness and processability, which are essential characteristic of a porous scaffold required for tissue engineering [28, 29]. It also possesses the ability to potentially maintain many cytokines, growth factors, and functional proteins that support the survival and proliferation of chondrocytes and the production and maintenance of cartilaginous matrices. Thus, PLGA allows the synthesis of extracellular matrix components as well as cellular structural support and can act as a biologically active agent to promote tissue regeneration. In particular, it has been extensively studied in the field of orthopedics. Total cartilage defects could be reconstructed with mesenchymal stem cell-PLGA scaffolds [30, 31]. In the field of otolaryngology, we reported successful partial tracheal defect reconstruction using allogenic chondrocytes cultured with fibrin/HA–PLGA construct in previous study [16]. In this investigation, PLGA can be used as grafts for augmentation rhinoplasty because it is a very useful biocompatible scaffold that can be easily applied compared to other synthetic materials.

This study demonstrated the positive results of *in vivo* implantation of fibrin/HA–PLGA scaffolds. *In vitro* experiments results showed more mature cartilage tissues after 8 weeks than those at 2 and 4 weeks. In addition, safranin-O staining confirmed that extracellular matrix production was remarkably increased after 8 weeks. During the 8-week observation period after implantation of the fibrin/HA–PLGA scaffold into the nasal dorsum of the animal model, gross evaluation did not show inflammatory response and the graft maintained an appropriate elevation level without any change in position. Because the augmented volume was mainly composed of fibrotic tissue with viable fibroblasts, further long-term effects could be expected, even though histological evaluation did not reveal the formation of neo-cartilage on the scaffold.

Although PLGA is already known as a biocompatible material, it is basically a foreign substance *in vivo*. Conventional artificial materials such as silicone and Gore-Tex® are excellent in outcome after augmentation rhinoplasty but cause foreign body reaction because they remain permanently in patient's body. In this experiment, PLGA degradation was observed with time. If cell proliferation and maintenance of extracellular matrix formation are accompanied, these properties of PLGA can be considered to be ideal materials of augmentation rhinoplasty. Therefore, in future experiments, the degradation of PLGA over time should be quantified. In addition, quantitative analysis of cell proliferation and extracellular matrix production will be necessary.

Despite the fact that the PLGA scaffolds showed promising results in this study, this approach might have some limitations in application to further augmentation rhinoplasty. Because we designed this experiment as a "proof-of-concept" study, we proceeded without a control group and observed the results at a single time-point *in vivo*. Also, we did not perform quantitative analysis on the results of this experiment. Therefore, long-term and larger animal studies involving control groups should be warranted prior to clinical application. Also, the tracing of implanted cells or amount of cartilage regeneration should be accurately measured in future studies.

Another limitations of this study are that in contrast to *in vitro* experiments, neo-cartilage formation and extracellular matrix production were not detected after 8 weeks of implantation *in vivo*. These results might be attributed to the use of allogenic chondrocytes in this investigation. Therefore, the use of autologous chondrocytes would be indispensable for future studies, since there is no rabbit study in which immunity is completely eliminated. In addition, in this study, we performed implantation of the scaffold on the supraperiosteum instead of the subperiosteum. This has a negative impact on the blood supply, causing insufficient supply of oxygen and protein necessary for neo-cartilage formation. Further studies will be needed to compare the results of cartilage formation according to the implant position of the scaffold.

This study evaluated the clinical efficacy of tissue-engineered structures for augmentation rhinoplasty. Considering the maintenance of planned shape and biocompatibility, PLGA scaffolds are considered as promising grafts for rhinoplasty. Further studies will reveal neo-cartilage formation, and if it is improved, major limitations in the currently performed autologous cartilage implantation may be resolved. In conclusion, the fibrin/HA–PLGA scaffolds are useful as an alternative material to overcome the limitations of implants for conventional grafts used in augmentation rhinoplasty.

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Compliance with ethical standards

Conflicts of interest The authors have declared no conflicts of interest.

Ethical statement This study was conducted under the approval of the Institutional Animal Experiment Committee at Ajou University School of Medicine (IACUC number: 2017-0023).

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