Evaluation of Diced Cartilage Grafts Shaped with Three-Dimensionally–Printed Bioresorbable Polycaprolactone Molds

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Background: The main problem with the use of diced cartilage grafts is related to the difficulties encountered in shaping the graft and unpredictible graft resorption. The aim of this study was to evaluate the permanence and viability of diced cartilage grafts shaped with the help of biodegradable, three-dimensionally–printed polycaprolactone molds.
Methods: Three groups were studied in each of the eight rabbits: block cartilage (group 1), diced cartilage (group 2), and diced cartilage shaped with polycaprolactone molds (group 3). A total of 24 cartilage grafts were obtained at the end of the 12-week follow-up period, and 10 different histopathologic parameters were analyzed in each cartilage graft.
Results: Diced cartilages shaped with a three-dimensionally–printed polycaprolactone mold showed increased regeneration potential of chondrocytes, vascularization, and collagen production. Use of polycaprolactone molds did not cause any additional risk of inflammation, fibrosis, or metaplastic bone formation.
Conclusions: In this study, it has been shown that three-dimensionally–printed polycaprolactone molds can be used safely in shaping diced cartilage grafts. In light of this study, it will be possible to produce hybrid grafts that can be used safely in many operations such as nasal reconstruction, rhinoplasty, auricle reconstruction, and repair of orbital floor fractures with the help of molds produced in more complex ways.
Clinical Relevance Statement: Three-dimensionally–printed polycaprolactone molds can be used to shape diced cartilages in the areas of both aesthetic and reconstructive surgery. (*Plast. Reconstr. Surg.* 150: 800e, 2022.)

Autologous cartilage grafts are frequently used in many operations as supportive material. However, concerns on the use of block cartilage grafts, such as the unpredictable amount of absorption, visibility under the skin, exposure from the skin, limited donor area, difficulties encountered in shaping the graft, and the need for revision because of evident bending, limit the use of these grafts.[1–5]

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Received for publication January 8, 2021; accepted December 20, 2021.

DOI: 10.1097/PRS.0000000000009572

These disadvantages of block cartilages emphasize the use of diced cartilage grafts. Erol[6] used the method known as "Turkish delight" to repair nasal dorsum deformities by wrapping diced cartilage grafts in Surgicel (Ethicon, Inc., Somerville, N.J.).[7,8] Daniel and Calvert applied this method by using a deep temporal fascia graft to solve problems related to chronic inflammation observed in patients.[9] To date, different alloplastic materials are used in clinical practice, but the search for an alternative material to shape diced cartilages continues.[10–12]

The use of polycaprolactone implants has gained popularity because of the recently developed three-dimensional printing technology, and use of the implants as an alloplastic implant

Disclosure: *The authors have no financial interest in any of the products, devices, or drugs mentioned in this article.*

material has been tried in many experimental studies[13–18] or as a tissue scaffold in autologous tissue engineering.[17–24] The idea of shaping diced cartilage grafts (which have much higher viability than do block cartilage grafts and a lower incidence of complications[25–28]) with molds produced in the desired shape and form was the starting point of this study. The study aimed to evaluate the effects of the use of polycaprolactone to shape autologous diced cartilage grafts on graft permanence and viability.

MATERIALS AND METHODS

A total of eight female New Zealand (*Oryctolagus cuniculus*) white rabbits aged 4 months and weighing 3000 to 3500 g were used in this study. Ethics committee approval was obtained for the subjects to be used in the study, and the use of experimental animals complied with the Declaration of Helsinki and the conditions proposed by the European Council (ETS123).

Design and Fabrication of Three-Dimensionally–Printed Bioresorbable Polycaprolactone Molds

To fabricate the 25 percent polycaprolactone scaffolds, polycaprolactone (2.5 g) was dissolved in dichloromethane (10 ml) at a concentration of 25 percent (weight/volume) under a magnetic stirrer for 1 hour at room temperature. After the homogeneous mixture was obtained, the solution was prepared to use in the three-dimensional printing system.

The polycaprolactone molds to be used in the study were produced with a three-dimensional printer (Ultimaker 2+; Dynamism, Chicago, Ill.) modified to work with the computer-aided design program Solidworks (Dassault Group, Vélizy-Villacoublay, France) and the G-code conversion program Simplify3D (Simplify3D, Cincinnati, Ohio). The needle diameter was set at 0.4 mm, extrusion rate at 0.8 percent, and default writing speed at 1 mm/minute. Polycaprolactone molds were produced in the form of 25 × 25 × 0.6-mm^3 square plates, and they were shaped with 5-0 Monocryl (Ethicon) sutures to form a 4-mm-radius cylinder with the help of an insulin injector.

Chemical, Morphologic, and Mechanical Characterization of Three-Dimensionally–Printed Molds

The morphologic structure of the molds used in the study was evaluated using a scanning electron microscope (EVO MA10; Carl Zeiss, Thornwood, N.Y.) twice: once before the implantation and once after graft harvest from rabbits in the 12-week follow-up period (Fig. 1). Before evaluation, the molds were covered with gold particles for 60 seconds with a coating machine (Emitech SC7620 Sputter Coater; Quorum Technologies, Lewes, United Kingdom). Fourier transform infrared spectroscopy (JASCO-4000; Jasco, Inc., Easton, Md.) was used for the physicochemical analysis of 25 percent polycaprolactone molds. To test the mechanical properties of the produced mold, a tensile test was performed using an electronic tension device (AGS-X; Shimadzu Corp., Kyoto, Japan). The speed of the test was set at 5 mm/minute, and three samples were tested to obtain an average value.

In Vivo Implantation and Harvesting

Three groups were studied for every eight animals to compare the viability of grafts under the same physiologic conditions. Each rabbit was subjected to general anesthesia by an intramuscular injection of 2% xylazine hydrochloride at 3 to 5 mg/kg and ketamine at 30 to 40 mg/kg. Local anesthesia was induced by 5% of 2 ml prilocaine to reduce the amount of ketamine induced and relieve postoperative pain. In each rabbit, two-thirds of the right distal part of the ear was amputated. The perichondrium of each ear cartilage was removed and a 25 × 7-mm^2 block of cartilage was harvested at the thickest portion of cartilage. The remaining cartilage was diced into 1-mm^3 pieces. Next, 1 ml of the diced cartilage grafts was randomly mixed with approximately 2 ml of blood obtained from the rabbit's ear. This fibrin-cartilage mixture was divided in two, and every 0.5 ml of the diced cartilage grafts was taken into 1-ml syringes with cutoff tips to form a cylindric shape. In group 2, cartilages were plumped very carefully into the donor area to avoid distorting their shape. In group 3, the polycaprolactone mold was folded over the second syringe and sutured with the help of a 5-0 Monocryl suture to create a tube and then filled with 0.5 ml of diced cartilage (Fig. 2). A total of three noninterconnecting pockets 1 × 3 cm in size in the subcutaneous plane of each rabbit's frontal area, one in the midline and two in the paramedian region, were planned. Block cartilage grafts were placed in the middle pocket (group 1), diced cartilage grafts were placed in the right pocket (group 2), and diced cartilage grafts shaped with polycaprolactone were placed in the left pocket (group 3) in all subjects. After the surgical procedure, 50 mg/kg intramuscular cefazolin injection was applied to all subjects once per day for 3 days.

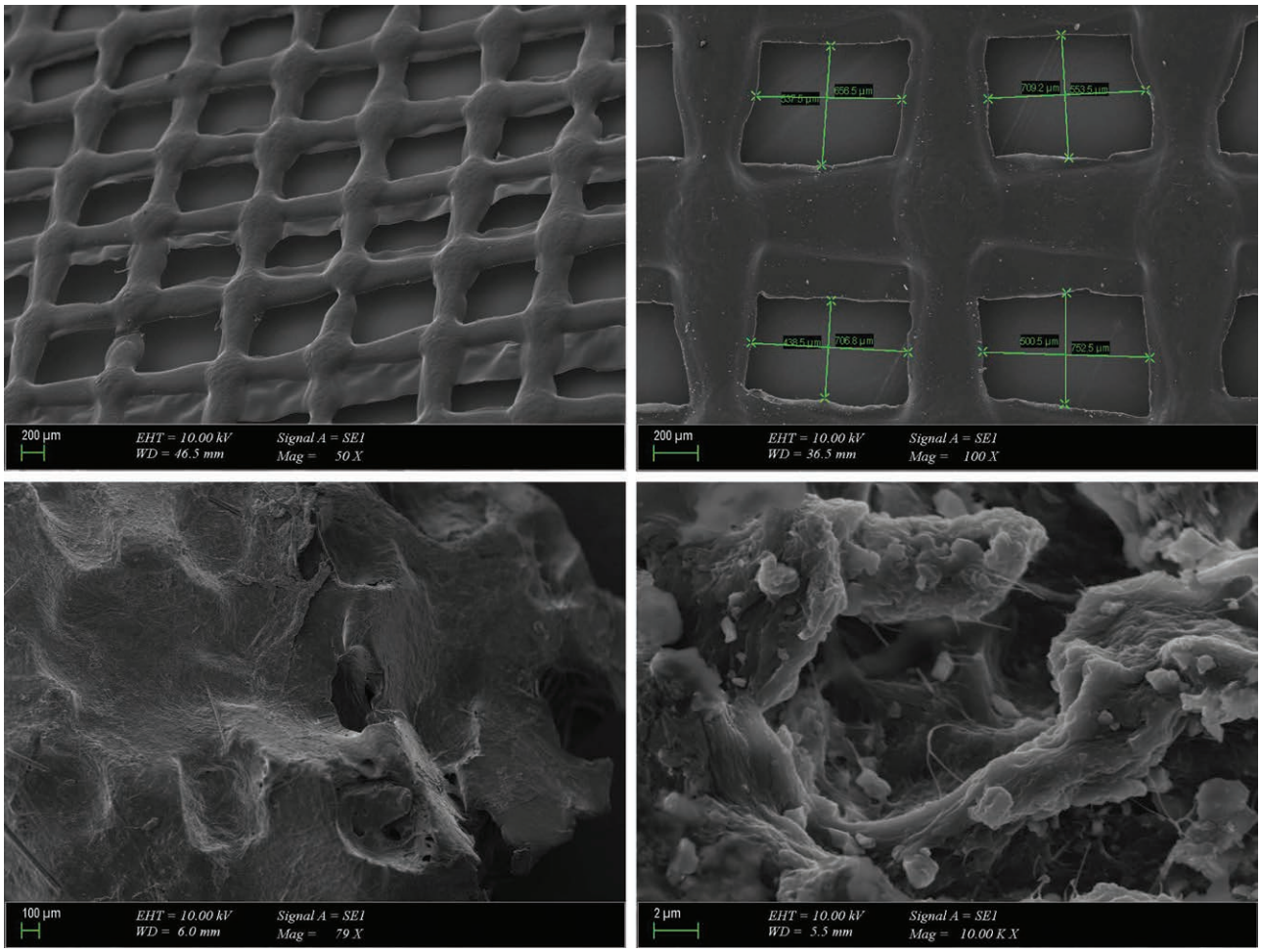

Fig. 1. Evaluation of polycaprolactone mold by scanning electron microscopy. (*Above*, *left*) Image of the prepared three-dimensionally printed mold at 50× magnification. (*Above*, *right*) After statistical analysis, the average pore size was found to be 608.66 ± 49 µm. (*Below*, *left*) As a result of 12-week follow-up, it was observed that the polycaprolactone mold was fully integrated with the cartilage (79×). (*Below*, *right*) Visualization of fibrovascular tissue around the pore structure (10.00 K×).

Sample Collection

The animals were euthanized with 100-mg/kg intramuscular ketamine injection at week 12. The skin over the grafts was incised, and the final state of the grafts, their shape, and their placement in their area were evaluated. Volume measurements of the isolated cartilage grafts were repeated and evaluated using a 10-ml measuring glass filled with water.

Histopathologic and Immunohistochemical Evaluation

After the measurements were completed, all the pieces were placed in 10% neutral formalin solution (pH 7.0) and fixed for 24 hours. After the fixation was completed, each piece was individually buried in a paraffin block. Five-micron-thick sections were taken from the paraffin blocks with the help of a microtome. Hematoxylin and eosin, Masson trichrome, Evans, van Gieson, and toluidine blue stains are used for histochemical examination. Glial fibrillary acidic protein stain was used for immunohistochemical examination, which demonstrates the regeneration potential of the chondrocytes. A brown deposition in the cytoplasm of a given cell indicates a positive staining for intermediate filaments. These filaments function as a part of the cytoskeleton and mechanotransduction system by which chondrocytes respond to external forces. This is considered to be evidence of the regeneration capacity of the cell.[29–31] Immunohistochemical staining was performed using an automated immunohistochemical system (Ventana Ultra; Ventana Medical Systems, Oro Valley, Ariz.), following the manufacturer's recommended procedures. The sections prepared were incubated with anti–glial fibrillary acidic protein (rabbit monoclonal antibody, EP672Y) as a glial cell marker. Subsequently, all sections were counterstained with hematoxylin dye.

Two pathologists blinded to the nature of the specimens performed the histologic analysis

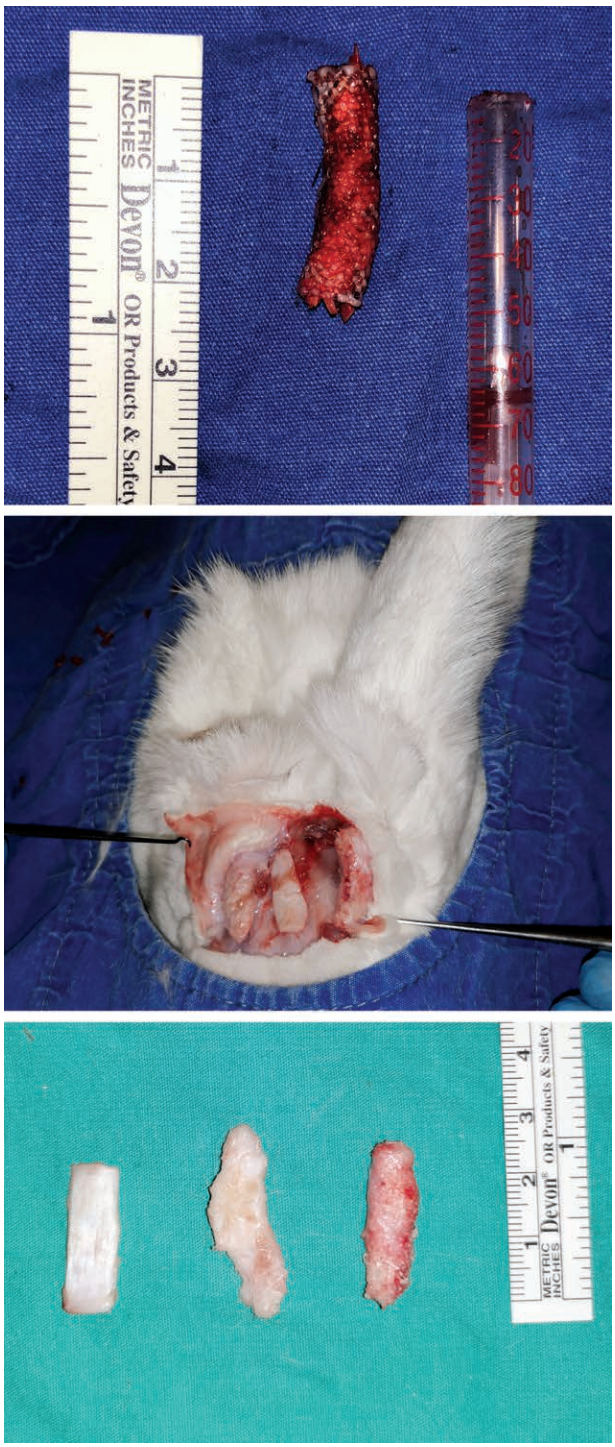

Fig. 2. (*Above*) Shaping the approximately 0.5 ml of diced cartilage grafts placed in a 1-ml injector with polycaprolactone mold into a cylindrical form. (*Center*) At the end of the 12-week follow-up period, the subjects were euthanized and the grafts were excised from the frontal region. (*Below*) Macroscopic views of the prepared grafts after 12 weeks of follow-up. (*Left* to *right*) Block cartilage graft, diced cartilage grafts, and diced cartilage grafts shaped with polycaprolactone mold.

according to a scoring system for evaluation of the morphology and histologic parameters per a procedure modified from Kim et al.[10] All slides were scanned at a magnification of 10×. Three groups of cartilage grafts were compared. A total of 18 fields were analyzed on two slides for each group. Ten different histopathologic parameters were evaluated in all three groups: chondrocyte nucleus loss and peripheral proliferation, chondrocyte regeneration potential, matrix collagen content, elastic fiber content, fibrosis, inflammation, vascularization, and metaplastic bone formation. Quantitative analysis of all parameters was conducted among the experimental groups. Parameters in the analyzed materials except glial fibrillary acidic protein staining were evaluated as percentages (0 percent = 0; 0 to 25 percent = 1+, 25 to 50 percent = 2+, 50 to 75 percent = 3+, and 75 percent and above = 4+). Glial fibrillary acidic protein staining was evaluated as 25 percent and below = 0; 25 to 50 percent = 1+; and 50 percent and above = 2+. All scores for a given specimen type were averaged and a two-tailed *t* test with unequal variances was used to compare the measured histologic characteristics for each graft type.

Statistical Evaluation

IBM SPSS Version (IBM Corp., Armonk, N.Y.) was used to compare the data in the study, and GPower (Version 3.1.9.4 for Windows; Heinrich Heine Universitat, Dusseldorf, Germany) was used to determine the power of the study. Categorical variables were defined with frequency and percentages; and continuous variables were defined with median, minimum, and maximum values. Kruskal-Wallis test was used for comparisons between groups, Mann-Whitney *U* test was used for paired comparisons of groups, and chi-square test was used for comparison of categorical variables. Fisher's exact test was used when the chi-square test was not appropriate. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Analysis of Polycaprolactone Mold

After macroscopic examination with scanning electron microscopy, the produced polycaprolactone mold was found to be almost the same as the design made with computer-aided design (Fig. 1). The polycaprolactone mold showed homogeneous and almost even pore distribution. The pore sizes of the polycaprolactone molds created were calculated with the SPSS program, and the average pore size was found to be 608.66 ± 49

μm. At the end of the 12-week follow-up period, the hybrid cartilage grafts obtained by euthanizing the animals were analyzed again by scanning electron microscopy, and it was observed that the polycaprolactone molds had some absorption but were completely integrated with the cartilage grafts. The chemical properties of the scaffolds were qualified by Fourier transform spectroscopy. There were five main absorption peaks observed at approximately 2940 cm, 2865 cm, 1720 cm, 1293 cm, and 1164 cm, which were related to asymmetric CH2 stretching, symmetric CH2 stretching, carbonyl stretching, C–O and C–C stretching in the crystalline phase, and symmetric COC stretching, respectively.[32] Nearly the same bulk polycaprolactone spectrum was obtained for the 25 percent polycaprolactone scaffolds.

The mechanical properties of these polycaprolactone scaffolds were evaluated through tensile testing. We observed three phases in the stress-versus-strain curve, which was the elastic region (linear deformation), stable stage (plateau), and final stage (permanent deformation).[33] The mean tensile strength value and elongation at the break percentage of the scaffolds were 11.95 ± 3.27 MPa and 263.11 ± 121.21 MPa, respectively. The tensile strength value (11.95 MPa) observed in this study is in the range of the articular cartilage, which is 1 to 20 MPa.[15–17] Normally, bulk polycaprolactone has a tensile strength value between nearly 10.5 to 16.1 MPa.[18] Although the printed scaffolds had a porous structure, their tensile strength was among the strength values of bulk polycaprolactone. The high strain value (263 percent) showed the high toughness of the scaffolds. The elastic modulus of the scaffolds was determined from the slope of the elastic region (1 to 5 percent elongation at break) and found to be approximately 3.2 MPa by using the average elastic modulus of the three measurements.

Macroscopic Analysis of Cartilage Grafts

All eight rabbits survived the 12-week experimental period without any complications. Group 1 preserved its structure and sharp lines, was easily separated from the surrounding tissues, and had natural cartilage elasticity. In group 2 and group 3, diced cartilage grafts were completely fused into a single cylindrical shape, with some hypertrophy of the grafts. The mean volume changes of groups 2 and 3 were significantly higher than that of the block cartilage graft group ($p = 0.001$). The surface of group 3 was smoother than that of group 2, and it preserved its cylindrical form more clearly. The polycaprolactone mold used in group 3 was mostly resorbed but fused with the cartilage; therefore, it could not be separated from the cartilage. Its porous structure was observed with careful inspection.

Results of the Histopathologic and Immunohistochemical Analyses

Ten different parameters were evaluated in all cartilage grafts (Figs. 3 and 4). There were no statistically significant differences between the groups in terms of nucleus loss and peripheral proliferation in chondrocytes showing the viability of cartilages. The regeneration capacity of chondrocytes and amount of collagen in group 1 and group 3 were significantly higher than in group 2. The polycaprolactone mold increased both chondrocyte regeneration capacity and collagen production in the diced cartilages.

In groups 2 and 3, in which the cartilage integrity was impaired, it was observed that metachromasia, fibrosis, and metaplastic bone formation were increased. There was no statistically significant difference in inflammation between groups 2 and 3. Vascularization was markedly increased in group 3 compared to groups 1 and 2 (Table 1).[34–36]

DISCUSSION

Diced cartilage grafts have much lower rates of resorption than block cartilage grafts.[25–27,37] This observation was previously explained by the formation of a much larger surface area in diced cartilages and increase in oxygen and nutrient diffusion as a result.[25,26] Another advantage of using diced cartilage grafts is that the fibroblasts migrating to the spaces between the cartilages that are broken down by the mincing process increase the amount of collagen and elastic fibers in the graft.[10] In our study, diced cartilages showed an increased amount of fibrosis and vascularization compared to the block cartilage group.

Problems related to the shaping of diced cartilages seem to have restricted the use of these grafts until the twenty-first century. Erol introduced the Turkish delight method, which can be summarized as shaping the diced cartilages with Surgicel.[6] However, Daniel and Calvert stated that the diced cartilage grafts formed with Surgicel could not maintain their shape for a long time and unforeseen cartilage resorption was observed in the short term.[9,38] This has been associated with the foreign body reaction that develops against Surgicel,[2,7,39,40] the loss of regeneration potential in chondrocytes because of relative hypoxia,[39] and the inability of the grafts to preserve the given

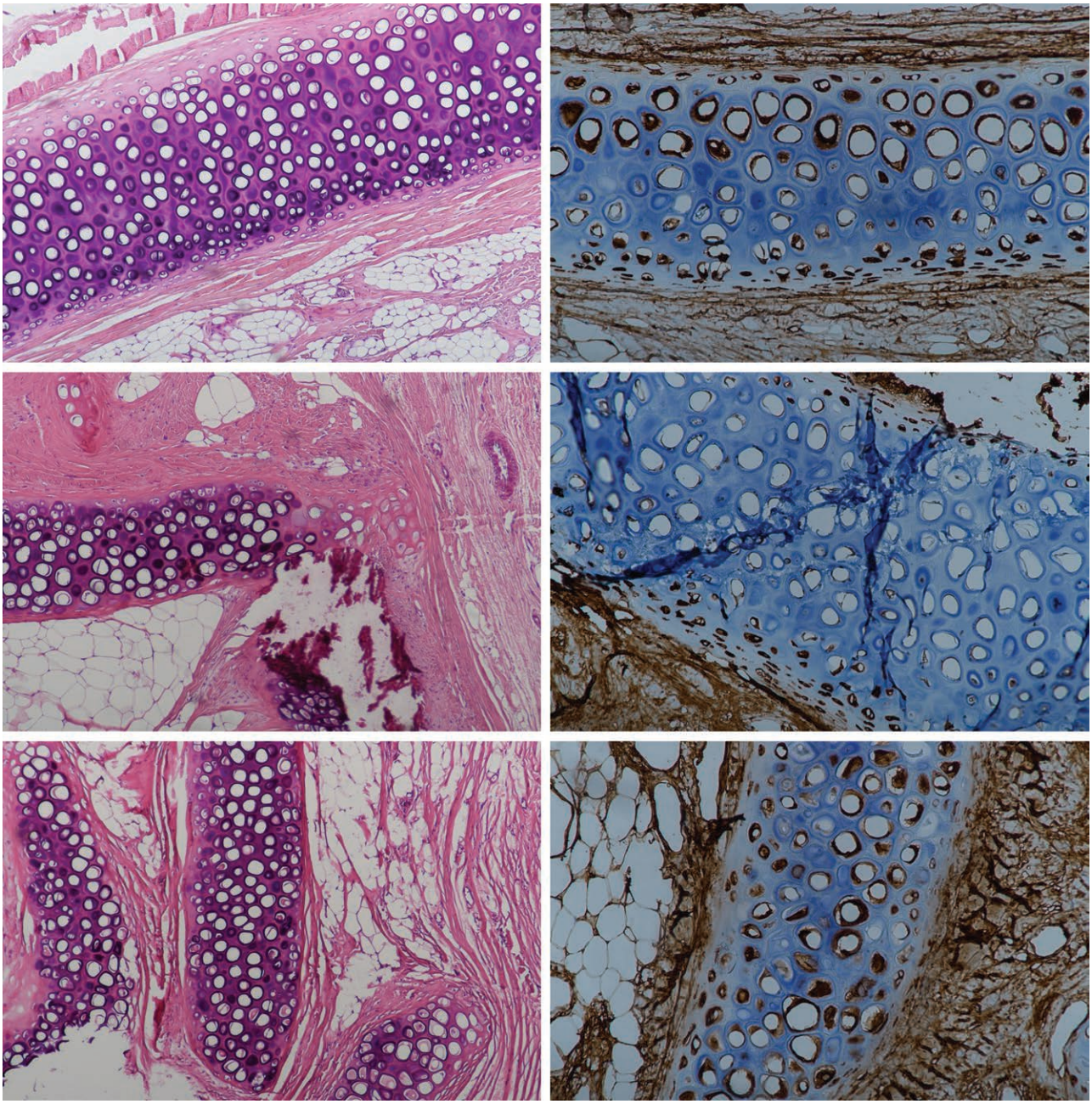

Fig. 3. Examination of grafts by hematoxylin and eosin and glial fibrillary acidic protein staining. Nucleus loss and peripheral proliferation in chondrocytes and vascularization, fibrosis, inflammation, and metaplastic bone formation in cartilage grafts were evaluated with hematoxylin and eosin staining (*left*). The regeneration capacity of chondrocytes was evaluated by staining with glial fibrillar acidic protein. Glial fibrillar acidic protein stains the intermediate filaments in chondrocytes dark brown (*right*). (*Above*) Block cartilage graft, (*center*) diced cartilage graft, and (*below*) polycaprolactone-shaped diced cartilage graft.

shape because of the absorption of Surgicel within 24 to 72 hours.[7,8,38,39] Daniel and Calvert suggested the use of autologous fascia instead of Surgicel and stated that cartilages shaped with autologous fascia graft showed a high regeneration potential.[9,28,29,41,42] Daniel and Calvert also stated that the autologous fascia that is used to wrap diced cartilages appeared to behave in a manner similar to perichondrium and had a role in facilitating chondrocyte survival, preventing graft absorption, and maintaining overall regenerative potential of the cartilage pieces.[38] Similar results were obtained with the use of polycaprolactone instead of autologous fascia in our study, which led to the conclusion that this polymer may have played the role of perichondrium for diced cartilage grafts.

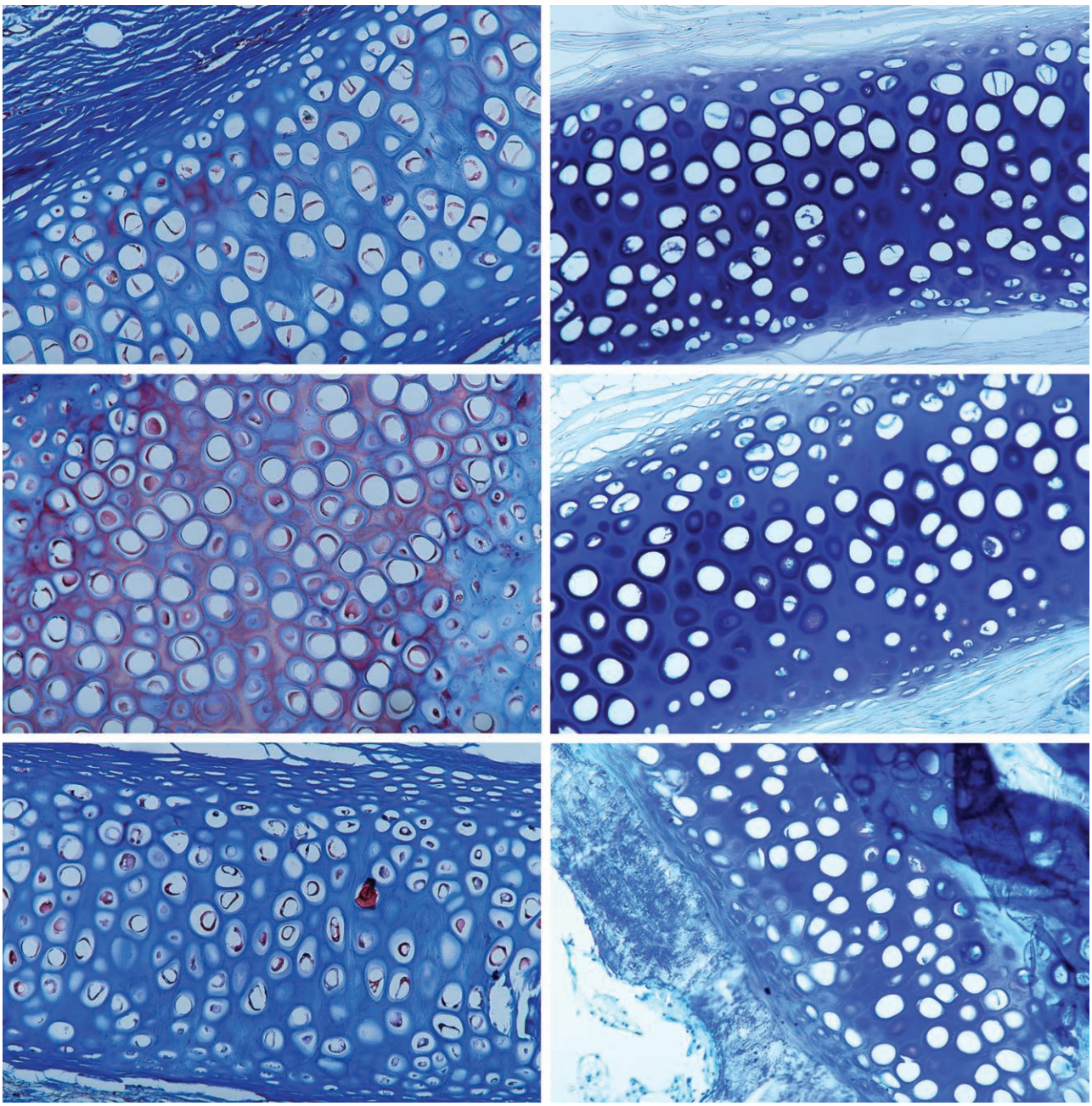

Fig. 4. Examination of grafts with Masson trichrome and toluidine blue staining. The collagen content in the matrix was evaluated by Masson trichrome staining (*left*). The amount of metachromasia in the grafts was evaluated by staining with toluidine blue (*right*). (*Above*) Block cartilage graft, (*center*) diced cartilage graft, and (*below*) polycaprolactone-shaped diced cartilage graft.

The higher amount of collagen and increased vascularization with marked increase in glial fibrillary acidic protein staining, which shows the regeneration ability of the chondrocytes, supports this theory.

Diced cartilages can become solid block cartilages with the desired shape if used with appropriate molds that can mimic perichondrium. For this purpose, the material to be produced in a three-dimensional printer should provide a suitable surface for the attachment of the cartilage graft; it should be biocompatible and bioresorbable; it should not prevent vascularization and new tissue formation while allowing serum imbibition with its porous structure; and it should provide adequate support without disturbing the integrity of the cartilage for the required period. Polycaprolactone is a biocompatible and biodegradable synthetic polymer with suitable mechanical strength and durability,[43–45] and it is frequently used in many fields of

Table 1. Statistical Comparison of 10 Different Histopathologic Parameters Evaluated between Three Graft Groups

Histopathologic Parameters	Block Cartilage Graft*	Diced Cartilage Graft*	PCL plus Diced Cartilage Graft*	*p*
Cartilages				
Nucleus loss	1 (0–1)	1 (1–2)	1 (1–2)	0.238
Peripheral proliferation	2 (2–3)	1,5 (1–3)	2 (1–3)	0.187
Regeneration potential	2 (2–3)	1 (1–3)	2 (2–3)	0.024†
Matrix				
Collagen	3 (3–4)	2 (1–2)	3 (2–3)	0.000†
Elastin	2 (1–3)	1.5 (1–2)	2 (1–3)	0.066†
Metachromasia	1 (1–2)	2 (2–3)	2.5 (2–3)	0.000†
Fibrosis	1 (0–1)	2 (1–3)	2 (1–2)	0.001†
Inflammation	0 (0–1)	1 (1–1)	1 (1–2)	0.003†
Vascularization	1 (1–2)	1.5 (1–2)	3 (2–3)	0.002†
Metaplastic bone formation	1 (0–1)	2 (1–3)	2 (1–3)	0.001†

IQR, interquartile range.
*Values are median (IQR).
†$p < 0.05$ (statistically significant).

medicine with U.S. Food and Drug Administration approval,[46,47] especially in tissue engineering.[19,20] Polycaprolactone safely breaks down into carbon dioxide and water and does not produce toxic metabolites. The hydrophobic nature of polycaprolactone and its ability to melt at temperatures above 60°C makes it possible to manufacture an implant designed specifically for the patient in a computer environment using three-dimensional printing technology. Although polycaprolactone's destruction period is approximately 2 years, the breakdown of byproducts of copolymers synthesized from ε-caprolactone and L-lactic acid begins in 4 to 6 months.[48] The polycaprolactone molds used in our study have a designed micropore structure that allows the removal of acidic metabolic wastes, which in turn allows cellular infiltration and tissue regeneration.[19] This micropore structure also helps the graft to integrate into the recipient area by facilitating fibrovascular tissue healing through these pores.[49] In our study, molds containing pores with an average size of 608.66 ± 49 μm were produced with 50 percent porosity, and biomechanical tests showed that these values can provide resistance to the cartilage tissue in the appropriate range as in autologous cartilage grafts.

There are many studies in the literature showing that polycaprolactone-based implants can preserve their structural integrity under various biomechanical loads in the long term.[13–16] Polycaprolactone has also been tested in animal nasal augmentation models, and successful results have been obtained.[17,18] In the study by Park et al. using polycaprolactone template as a septal extension graft in a rabbit model, dense fibrovascular growth was observed from the pores at the end of 3 months, and minimal inflammatory response was observed.[17] Wiggenhauser et al. studied three-dimensionally–printed polycaprolactone implants used for nasal dorsum augmentation in guinea pigs and observed no foreign body reaction or infection.[21] In many cartilage engineering studies where polycaprolactone is used as a tissue scaffold, it has been shown that polycaprolactone supports cell attachment and proliferation and the formation of the extracellular matrix for chondrocytes.[20,22–24] This finding indicates that polycaprolactone is not only a support material but also creates a suitable microsystem for cartilage regeneration.[49]

There are only three studies in the literature that aim to give complex three-dimensional shapes to diced cartilage grafts with molds. Peer used a Vitallium ear mold to shape diced cartilages into ear form.[28] Pomahac et al. crushed costal cartilage grafts and shaped them with poly-L-lactic/polyglycolic acid (LactoSorb; Biomet Microfixation, Dordrecht, The Netherlands) mesh in S and U shapes. They obtained cartilage tissue in the desired shape in 8 weeks.[50] Liao et al. shaped diced cartilage grafts with a nonabsorbable polyamide ear mold produced in a three-dimensional printer and showed that cartilages have high viability at the end of 4 months. However, it was observed that a fibrocystic capsule containing serous exudate was formed around the grafts. Although this situation did not cause a problem for chondrocyte viability, the long-term complications of this situation, which is thought to develop because of a foreign body reaction, cannot be predicted.[51]

We used a biocompatible and biodegradable material (i.e., polycaprolactone) in our study. This is the first study in the literature in which diced cartilage grafts were shaped with three-dimensionally–printed polycaprolactone molds that were produced very finely to mimic the function of fascia grafts. Its application was studied in a simple way by turning

it into a cylinder with the help of sutures. No statistically significant difference was observed between the diced cartilage grafts shaped with polycaprolactone and diced cartilage grafts in terms of metachromasia, fibrosis, inflammation, and metaplastic bone formation. Vascularization, collagen amount, and regeneration potential of chondrocytes were found to be statistically significantly higher in cartilage grafts shaped with polycaprolactone compared to bare diced cartilage grafts.

CONCLUSIONS

Diced cartilage grafts can be shaped with the help of polycaprolactone molds, which can be designed and produced in office settings. Polycaprolactone might be the ideal alloplastic material for shaping diced cartilages because of its biocompatible and biodegradable nature with suitable mechanical strength and durability. It is possible to produce specifically shaped hybrid grafts with the help of three-dimensional printing technology to be used in operations that need cartilage grafts, such as rhinoplasty, nasal reconstruction, auricle reconstruction, and orbital floor fracture repair.

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