

Histological and Immunohistochemical Analyses of Repair of the Disc in the Rabbit Temporomandibular Joint Using a Collagen Template

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Abstract

Previous study demonstrated the reconstituted type I collagen matrix extracted from rabbit tendons enabled to regenerate the TMJ disc in the rabbit. The aim of this study was to investigate changes in the extracellular matrix (ECM) and mechanisms of regeneration in TMJ disc. In 36 New Zealand rabbits that underwent a partial discectomy, discs were replaced with reconstituted collagen templates for 3 months. A histological analysis showed that moderate to severe degeneration appeared in partially discectomized joints without implantation. In contrast, discs that received the reconstituted collagen template regenerated, and returned to normal to protect the joint. Cells in the regenerative tissue expressed ECM, and fibers became regular and compact due to tissue remodeling over time. Reparative cells differentiated into chondroblasts, and showed highly dense pericellular fibers. The morphology and collagen composition of the disc and condyle in the 3-month experimental group were similar to those of normal tissues. In conclusion, the reconstituted collagen template facilitated the regeneration of surgically discectomized discs. Type I and type II collagens play a crucial role in the regeneration of articular discs.

Key word: temporomandibular joint disc, reconstituted collagen template, tissue regeneration.

Introduction:

Temporomandibular joint (TMJ) disorders (TMDs), major causes lead to degenerative changes in the disc and condylar cartilage. If pathologic changes persist following conservative or non-surgical treatment [1], degeneration or perforation of the disc might occur, and followed by a surgical removal of the disc [2, 3]. Surgical techniques such as a discectomy, arthroplasty, and condylectomy have been used to provide migration of blood cells and mesenchymal stem cells from the bone marrow to the defect [4]. These surgical treatments achieve fibrocartilagenous healing that may improve symptoms; however, the reparative tissues often cause limitations of joint function [5].

Previously, we designed a reconstituted collagen template for cartilage regeneration of the rabbit TMJ disc after a partial discectomy [6, 7], and established a model of *in vitro* proliferation of chondrocytes in a collagen matrix [8, 9].

Hematoxylin and eosin (HE) staining results showed that the template induced regeneration of the articular disc. However, the mechanism of disc regeneration is still not well understood. Also given the scarcity of studies morphologically focusing on the types of collagen in TMJ disc regeneration, a better understanding structural mechanisms between collagens and disc regeneration of the TMJ is well demanded.

The purpose of this study was to further determine the collagen matrix pattern of the regenerative disc and joint cartilage in experimental discectomies of the TMJ in rabbits. Immunohistochemical evaluation of discectomized discs, repaired using a reconstituted collagen template, was employed. Results showed a similar collagen distribution to that of normal joint tissue.

Materials and Methods:

Rabbits

Thirty-eight adult New Zealand male rabbits were housed in well-ventilated cages and fed a regular diet. The average age of the rabbits was 3 months, and their average weight was 2.0 kg. All animal husbandry and handling procedures including animal monitoring, diet, primary enclosures, and environmental control followed standard operating procedures in accordance with the *Animal (Scientific Procedures) Act* of 1986. Ref. All animal experiments were approved by the Animal Care Committee of Taipei Medical University (LAC-92-0036).

Reconstituted collagen templates

Reconstituted collagen templates were prepared as previously described [6, 7]. Type I and type II collagens were extracted and purified from tendons and cartilage of New Zealand white rabbits as previously described in our laboratory [10].

Experimental design

Thirty-eight animals were divided into experimental (collagen template implantation) (n=18), untreated (without implantation) (n=18), and sham-operated groups (n=2). The experimental and untreated groups comprised 18 animals in each group: 6 animals under observation for 3 months, 6 animals for 2 months, 6 animals for 1 month, respectively, after the partial discectomy. The remaining 2 animals comprised the sham-operated group. The unoperated sides served as intact controls.

Surgical techniques

Thirty-six adult New Zealand male rabbits underwent a partial posterolateral discectomy of the TMJ disc. Two sham-operated rabbits were only opened up and closed without specific removal of joint tissue. The TMJ region of each rabbit was shaved and prepped with povidone-iodine solution under general anesthesia with ketamine (35 mg/kg) and Citosol (50 mg/kg), followed by lidocaine infiltration. Half of the operations were performed on the right side and the other half on the left side to avoid the operation side as a confounder in the biostatistics.

A curvilinear incision was made along the zygomatic arch extending from the lateral aspect of the canthus to just anterior to the external auditory meatus [7]. The overlying tissue was flapped inferiorly, and the TMJ was exposed. Following the incision along the articular fossa and the eminence, a 0.5-cm segment of the

zygomatic process was removed. The capsule tissue was reflected, and the disc was identified.

A partial discectomy (3.5×6.0 mm) was performed on the posterolateral portion of the TMJ disc. The reconstituted collagen templates or dermal grafts were immediately implanted as the disc-replacement and fixed, after which the articular capsule was closed with 4-0 silk non-resorbable sutures. The skin incision was then closed with 4-0 silk non-resorbable sutures.

After surgery, the body weight of each rabbit was measured weekly to determine whether clinical problems in the TMJ were reflected by food intake. From 1 to 3 months after surgery, animals were sacrificed by a lethal intraperitoneal pentobarbital injection (60 mg/kg); the TMJ tissue with implants was excised en bloc and processed for gross, histological, and immunohistochemical (IHC) evaluations.

Histology preparation

TMJ tissues with implants were coronally en bloc excised. Specimens were fixed in formalin, and decalcified with DECAL-RAPID (National Diagnostics, Atlanta, GA) for 10 h. Tissues were then embedded in paraffin and serially sectioned (Sakura Sledge microtome, Sakura Finetek Japan, Tokyo, Japan) at 5~10 μ m. Tissue sections were stained with HE. The tissue regeneration and/or fibrosis of the defective

area were evaluated histologically including host response, tissue response to the surgical trauma, and tissue regeneration.

Immunohistochemistry and relative quantification

Serial sections of each sample were incubated with the first antibody (anti-collagen type I and II) or control blank serum. The antigen-antibody was further incubated with a horseradish peroxidase secondary antibody. Complexes were revealed with diaminobenzidine (DAB) to determine the collagen typing changes. The expression levels of type I, and II collagens were evaluated using relative immunochemical staining TMJ disc tissue sections, which compare between different samples based on objective data [11]. Images were acquired sequentially by Aperio Scanscope Console software (Informer Technologies, Inc., Shingle Springs, CA). TIFF images were processed using Photoshop 4.0 software (Adobe).

Statistical analysis

The expression levels of type I, and II collagens were analyzed using at least three samples and counted for each of the two groups. Statistical computations were performed using Student *t* test. Data are reported as the mean \pm S.D. $P < 0.05$ was considered statistically significant.

Results:

Sham-operated group

Histological evaluation

The normal articular disc was coronally concave in shape and composed of dense connective tissue. Synovial fibroblasts and chondroblasts were found in the dense connective tissue.

The condyle was composed of cancellous bone covered by a thin layer of compact bone. The outer surface on the condyle was covered with fibrocartilage tissue (Fig. 1C). Between the fibrocartilage layer and compact bone was a layer of hyaline cartilage (Fig. 1C). The fibrocartilage layer consisted of a layer of fibrous tissue with scattered chondroblasts (Fig. 1C, D). No significant difference between the unoperated and sham-operated groups was found. The TMJ disc was composed of wavy and thick collagen fibers where disc chondroblasts were growing (Fig. 1D).

IHC evaluation

In normal TMJ cartilage, type I collagen was expressed on all fibrocartilage layers, and type II collagen was mainly expressed in the hypertrophic zone (Fig. 1A, B). In the disc, type I collagen was revealed around wavy fibers (Fig. 1 E, G). Type II collagen was expressed around clusters of polygonal chondroblasts in the outer part of

the disc (Fig. 1F, H). Approximately, the ratio of collagen expression of type II to type I is 1.5 to 1. (Fig. S1)

Untreated group

Histological evaluation

All 18 untreated discectomized specimens were evaluated after 1 month, 2 months, and 3 months. Six discectomized joints in 1-month group showed mild to moderate fibrosis on the condyle and the remainder of the disc at 1 month. Cartilage erosion and enlargement were found on the outer layer of the condyle and tympanic fossa. Degeneration was noted in the disc. Chondroblast proliferation and cluster formation were predominantly found in the outer layer of cartilage in both the condyle and tympanic fossa (Fig. 2A).

In 2 month-group, the condyle and tympanic fossa showed moderate erosion which was accompanied by marked cartilage enlargement and fragmentation in whole group of six animals. Chondroblast proliferation was scattered in the cartilage (Fig. 3A). Discs and condyles showed moderate to marked fibrosis with degeneration.

Cartilage was completely eroded from the subchondral bone in the 3-month group. The remainder of the disc was torn out, and the articular surface was covered with fibrous connective tissue in five of six joint specimens (Fig. 3B). The sixth joint

exhibited severe cartilage erosion of the condyle. All six joints exhibited severe erosion of the tympanic fossa.

IHC evaluation

Type I collagen showed an increase on the cartilage surface and in the zone of chondroblast proliferation. The layer of hyaline cartilage expressed abundant type I collagen than that expressed in normal specimens 1 month or 2 months after discectomy (Figs. 2B, 3C). An increase in type II collagen around proliferating chondroblasts was also found in the cartilage area of the mandibular condyle process and tympanic fossa in the arthritic group (Figs. 2C, 3D). A different appearance found in the remainder of the disc compared to the condyle. Type I collagen did not change (Figs. 2D, 3E); however, type II collagen showed only minimal (Figs. 2E, 3F). The partial discectomized joint without implantation exhibited obviously inflamed in 2 months. Type I collagen had remarkably increased in both the enlarged and fragmented portions of the condyle, whereas type II collagen showed a mild increase in the condyle and was almost not found in the disc. The relative quantification of collagen expression showed parallelly to the immunohistology in the disc. Almost no change of type I collagen expression in 1 month, and a significant increase of type I

collagen expression in 2 month. Minimal expression of type II collagen was noted in the disc either in 1 month- or 2 month- groups. (Fig. S1)

Surgery with implantation

One-month group

Histological evaluation

A mild regeneration from the medial side laterally and covered one-third of the condyle in the implant group was shown in Figure 4 A. The regenerated part appeared thinner and more primitive compared to the initial part. No disc ossification or calcification was observed in discs of either the experimental or control groups. The condyles of the experimental group showed mild erosion, whereas the control group showed mild to moderate erosion. No cartilage fragmentation was observed in the condyles of the experimental group and control group.

The remainder of the reconstituted collagen template was noted at the surgical site. Lymphohistiocytic infiltration and foreign body giant cells were also present.

IHC evaluation

Mild type I collagen expression was found on both the condyle and disc (Fig. 4B, D, F). However, less type I collagen was noted compared to the 1-month group

without collagen template implantation. Type II collagen had not obviously changed in the cartilage or disc (Fig. 4C, E, G). The quantification of expression appeared a mild decrease both in type I and type II collagen (Fig. S1). The template showed a protective capacity in the 1-month group.

Two-month group

Histological evaluation

Disc regeneration covering more than one-third of the condyle was noted. The regenerated disc of the 2-month group appeared a little thicker than that of the 1-month group (Fig. 5A). No fibrosis, ossification, or calcification was found on the disc (Fig. 5A). No fibrous layer erosion was observed on the condyle or the tympanic fossa of the temporal bone.

IHC evaluation

The collagen pattern was almost the same as that of normal specimens in both the joint cartilage and disc (Fig. 5B, C). The template and regenerated disc prevented tearing of the cartilage and inflammation of the joint.

In the remainder of the disc expressed type I collagen fibers as wavy appearance (Fig. 5D), and expressed type II collagen around chondroblasts (Fig. 5E,

G). This pattern of the 2-month group was similar to that in the intact control group. Type I collagen was also expressed in cells at the cutting surface of the disc (Fig. 5F). The ratio of type I collagen to type II collagen in the disc seemed to become normal, although the amount of collagen showed approximately half of the intact control (Fig. S1). These indicate that some newborn cells had repaired or regenerated the injured disc by synthesizing type I and type II collagens. These results indicate that the template can diminish tearing of the joint and promote regeneration of the disc.

Three-month group

Histological evaluation

Discs of the experimental group showed apparent regeneration 3 months after implantation (Fig. 6A). New collagen bundles had formed and adhered to the tympanic fossa. Primitive chondrocyte-like cells were scattered in mature collagen bundles. New collagen bundles appeared slightly disoriented compared to the normal (Fig. 6B).

IHC evaluation

After 3 months of implantation, the regenerated disc had almost covered the TMJ cartilage with a normal collagen pattern and layers (Fig. 6C, D). Type I collagen

of the regenerated disc was revealed on collagen bundles (Fig. 6E), and type II collagen was expressed around fiber sections and chondrocyte-like cells (Fig. 6F). The ratio of type I to II collagen is 1.2 to 1, which is close to the intact control (Fig. S1). The collagen pattern was nearly same as normal tissues.

Discussion:

Dermal grafts can relieve the pain of TMDs and provide protection to maintain cartilage integrity [12, 13], although they are insufficient to induce the regeneration of the disc [14]. Since 1989, temporalis muscle flaps have been the usual treatment for TMDs [15, 16], somewhat like derma grafts, which provide no disc repair. In our study, the reconstituted collagen template formed optimal 50~150- μ m pores that were suited for cell ingrowth [17]. Attempts have been made to apply stem cells [18, 19], cytokines [20-22], platelet-rich plasma [23] for TMJ regeneration, however, no predictable therapy is currently available in the clinic, thus our implants provide a method to regenerate TMJ discs. In the beginning of wound repair, reconstituted collagen matrix provided mechanical resistance to protect the cartilage. Then, the pores and scaffold of the template recruited cell ingrowth and connected with the remaining disc. These double facilitation of the collagen template increase the disc regeneration better than other traditional implants.

Immune responses cause major problems for exogenous implant applications [24]. Our previous study reported a reconstituted collagen template lasted 3 months in vivo [7]. Lymphohistocytic infiltration and foreign body giant cells were found within the collagen template in the first 2 months. This immune response ceased with complete degradation of the template in the third month. In the present study, the inflammatory response induced by the exogenous template did not cause obvious cartilage destruction, although inflammation was noted in the beginning of TMJ osteoarthritis. The reconstituted collagen template is not only required to remain long enough until complete disc regeneration, but it also moderates exogenous material-induced inflammation.

The amount collagen loss and synthesis play the crucial role in the osteoarthritic change or repair. Our data showed that once the collagen gradually lost in 1 to 2 months, the TMJ became totally destroyed in 3 months. In contrast, the TMJ disc turned to regeneration, if the collagen matrix rebuilt up. This indicates that type I and type II collagens play a crucial role in the regeneration of articular discs.

Recently, Brown et al. used porcine urinary bladder matrix (UBM) as a disc material. Results showed TMJ protection and disc regeneration [25, 26]. UBM is mainly comprised of type I collagen and has similar components as the reconstituted collagen template [27, 28]. In the canine TMJ, UBM protected TMJ cartilage and

triggered cell and vessel ingrowth to form new disc tissues after 6 months of treatment [25, 26]. The UBM and reconstituted collagen template demonstrate that acellular ECM-based matrices facilitate disc regeneration in the TMJ. However, UBM, like the reconstituted collagen template, also induced a foreign body reaction [25, 26]. Long-term inflammation needs to be moderated and controlled, although the immune response decreased with time.

Autogenous grafts, such as temporalis flaps [15, 29], auricular cartilage [30, 31], and dermis-fat grafts [13, 32], have been used in animals with a discectomy and have shown varied success rates. However, they require more surgery and have no inductive effect on disc regeneration. Alloplastic materials, like sialastic [33] and Teflon [34] implants, have been withdrawn because of severe foreign body reactions and bone erosion [33, 35]. Recently, exogenous acellular ECM-based materials [36], such as reconstituted collagen templates and UBM, have shown more predictable regenerative capacities compared to other implants. If immune activation by reconstituted collagen templates can be resolved and biodegradation can be controlled until the completion of disc regeneration, acellular ECM-based materials will be potential candidates for TMD therapy.

In summary, the reconstituted collagen template protected TMJ cartilage from trauma and had induced disc regeneration 3 months after a discectomy. With

reconstituted collagen templates, TMJ cartilage maintained normal collagen expression, and recruited disc cells and chondrocyte-like cells that expressed type I and type II collagens respectively to regrow the excised disc. The reconstituted collagen template can be a potential candidate for implantation for TMD therapy, although immune activation needs to be controlled. Further studies will focus on controlling the biodegradation rate and alleviating immune responses.

Conflict of interest

None declared.

Acknowledgements

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Figures and Legends

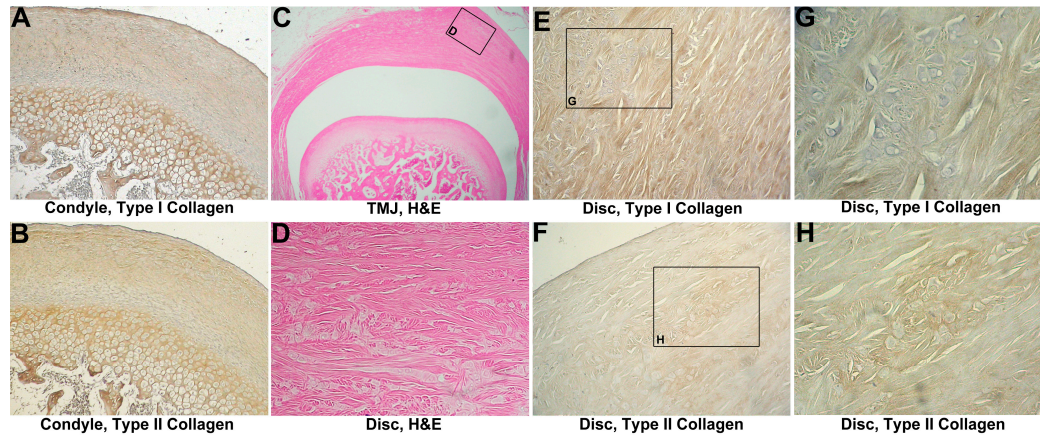


Figure 1. Analysis of the intact control. (A) Immunohistochemistry of type I collagen on normal temporomandibular joint (TMJ) cartilage, 800x. Type I collagen was expressed on the cartilage surface, bone, and proliferation zone. (B) Immunohistochemistry of type II collagen on normal TMJ cartilage, 800x. Type II collagen was expressed at the hyaline zone and proliferation zone. (C) HE analysis of a normal TMJ, 160x. Normal architecture of the disc, condyle, and cartilage. (D) HE analysis of the normal TMJ disc, 1600x. Chondrocytes, surrounded by wavy fibers, are scattered in the disc. (E) Immunohistochemistry of type I collagen in the normal TMJ disc, 1600x. Type I collagen was noted in wavy fibers. (F) Immunohistochemistry of type II collagen on a normal TMJ disc, 1600x. Type II collagen was expressed around chondrocyte-like cells. (G) Immunohistochemistry of type I collagen in the normal TMJ disc, 3200x. Magnification of (E). (H)

Immunohistochemistry of type II collagen in the normal TMJ disc, 3200x.

Magnification of (F).

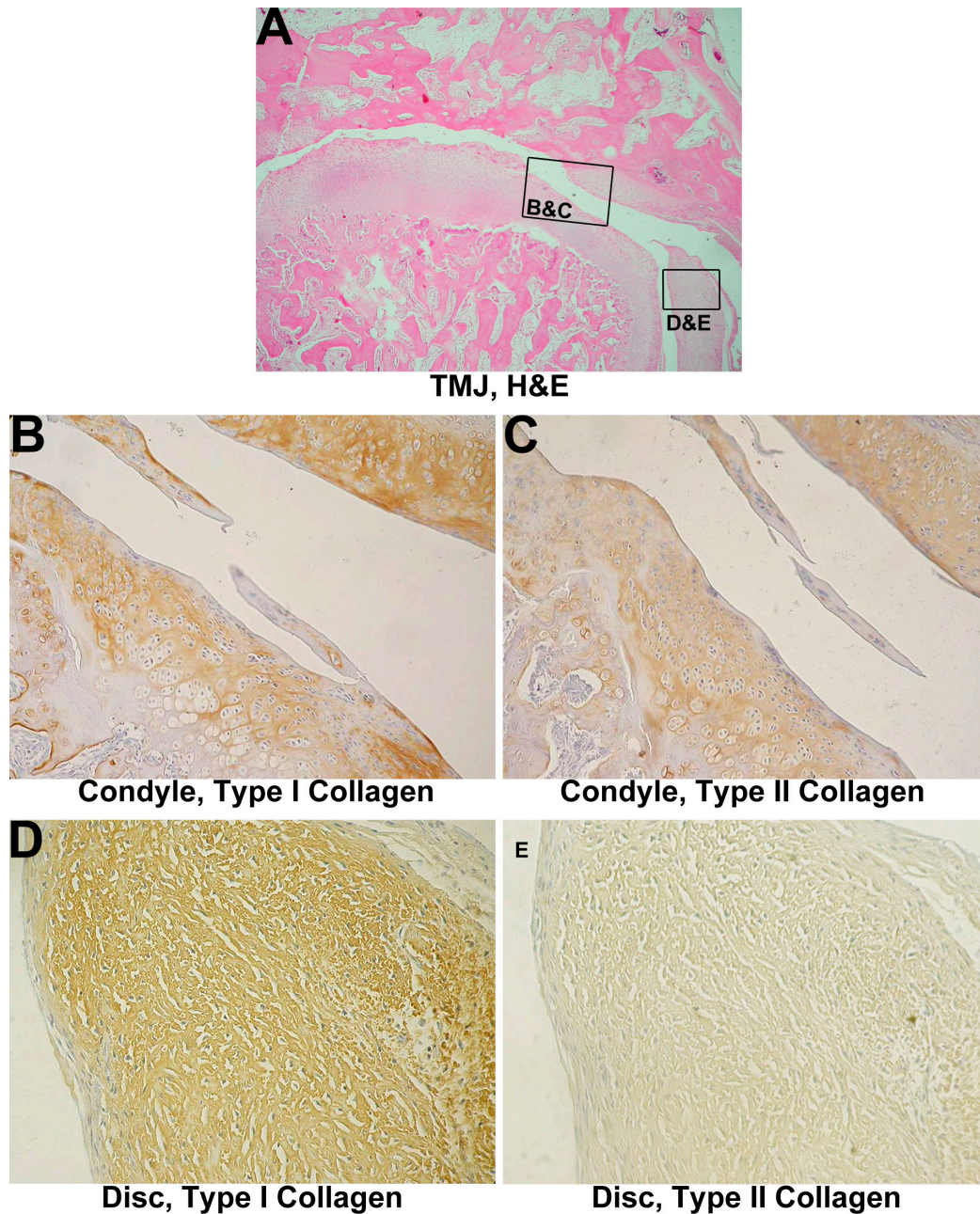


Figure 2. Histological analysis of a 1-month discectomized TMJ without implantation.

(A) One-month group, 320x. Mild chondroblast proliferation in the cartilage was evident. The condyle surface appears erosive and moderately deformed. The remainder of the disc shows fibrous degeneration. (B) Immunohistochemistry of type I collagen on 1-month untreated TMJ cartilage, 800x. Type I collagen expression

increased and surrounded proliferating cells on the cartilage surface. (C)

Immunohistochemistry of type II collagen on 1-month untreated TMJ cartilage, 800x.

Type II collagen, like type I, surrounded proliferating cells. (D)

Immunohistochemistry of type I collagen on a 1-month untreated TMJ disc, 1600x.

Type I collagen showed nearly normal expression. (E) Immunohistochemistry of type

II collagen on a 1-month untreated TMJ disc, 1600x. Minimal expression was

observed.

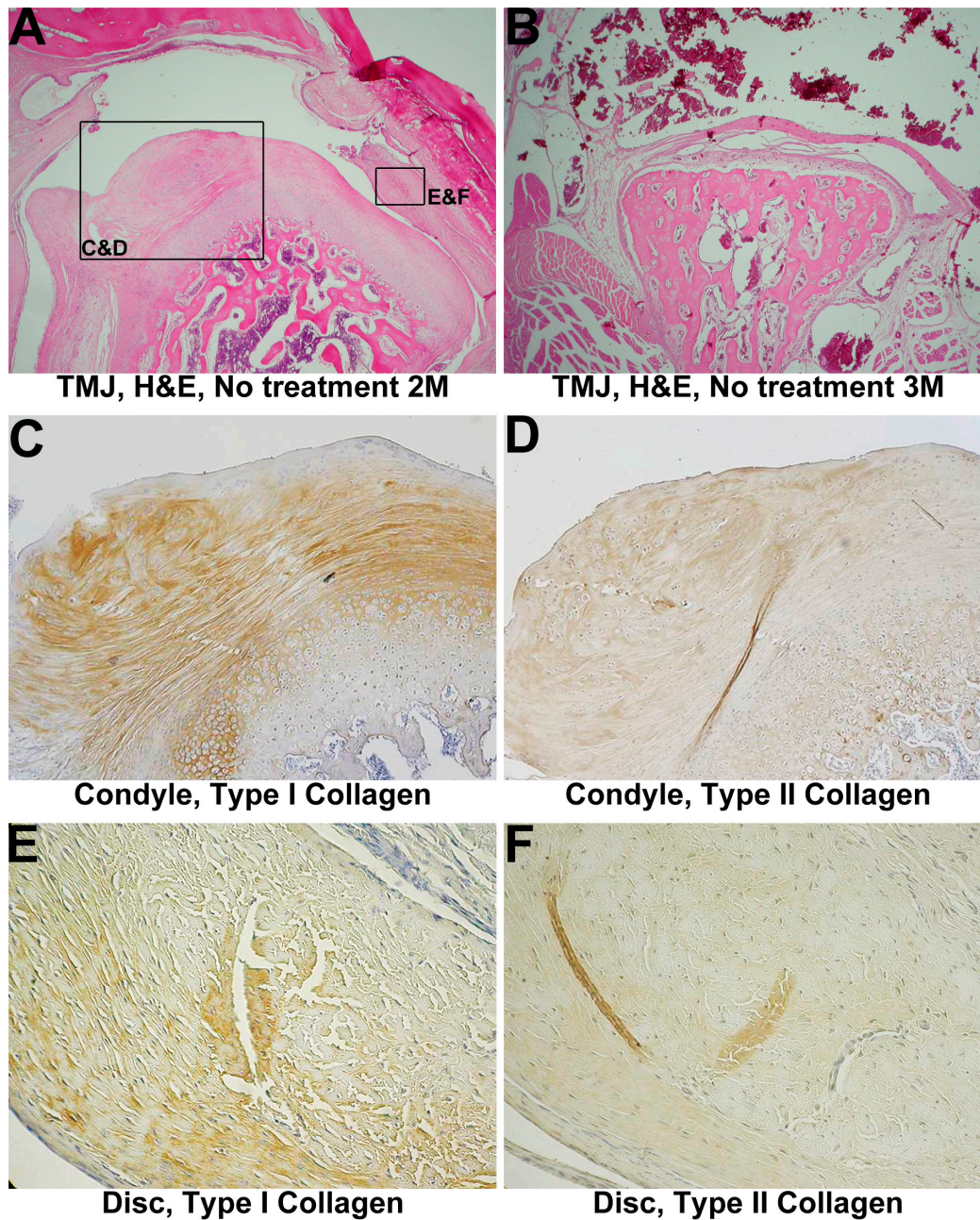
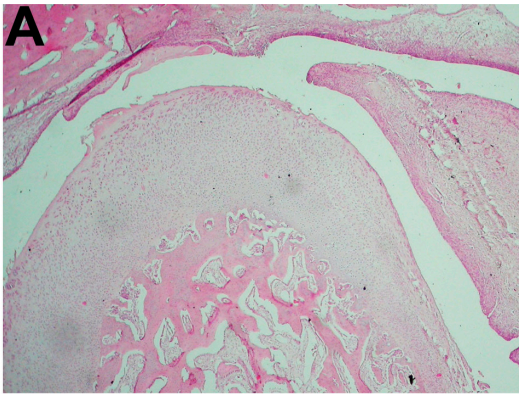
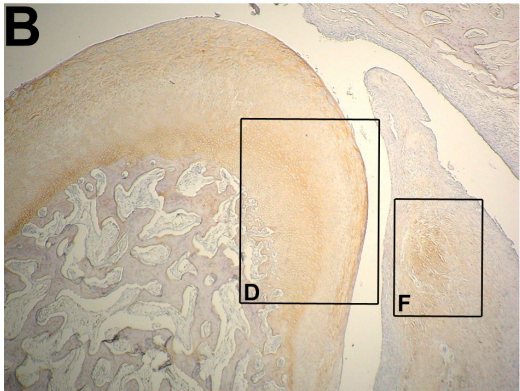


Figure 3. Histological analysis of the discectomied TMJ without implantation. (A) Two-month group, 320x. The remainder of the disc is characterized by fibrillary degeneration, and it was covered by a synovial lining. Marked cartilage enlargement and fragmentation, and fibrous degeneration of both the condyle and tympanic fossa was found. (B) Three-month group, 320x. The condyle shows complete erosion of

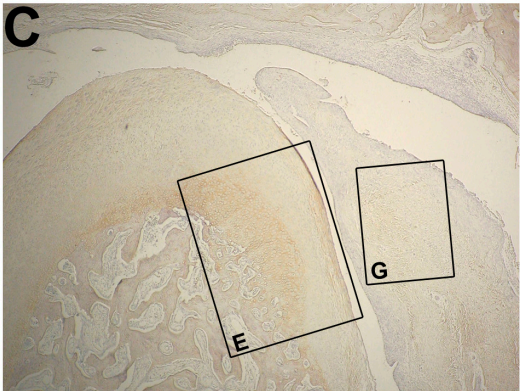
cartilage from the subchondral bone. (C) Immunohistochemistry of type I collagen in 2-month untreated TMJ cartilage, 320x. Type I collagen was greatly expressed in enlarged cartilage. (D) Immunohistochemistry of type II collagen in 2-month untreated TMJ cartilage, 320x. The expression of type II collagen had significantly decreased in enlarged cartilage. (E) Immunohistochemistry of type I collagen in a 2-month untreated TMJ disc, 1600x. Like the normal disc, type I collagen expression was found in fibers. (F) Immunohistochemistry of type II collagen in the 2-month untreated TMJ disc, 1600x. Minimal type II collagen expression surrounds cells.



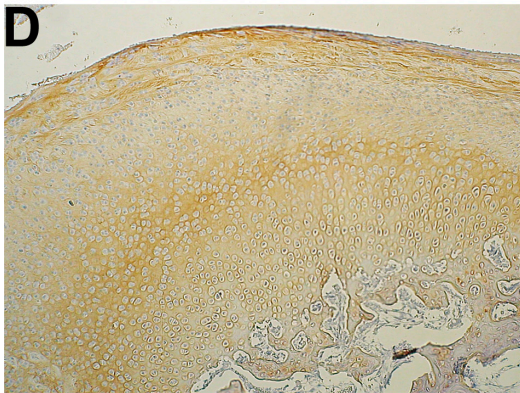
TMJ, H&E



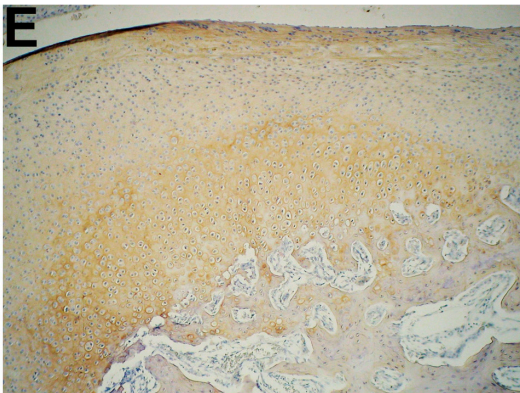
TMJ, Type I Collagen



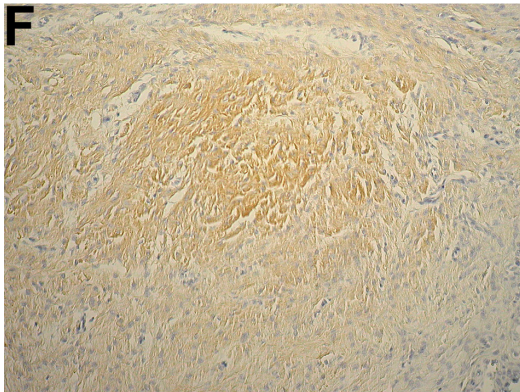
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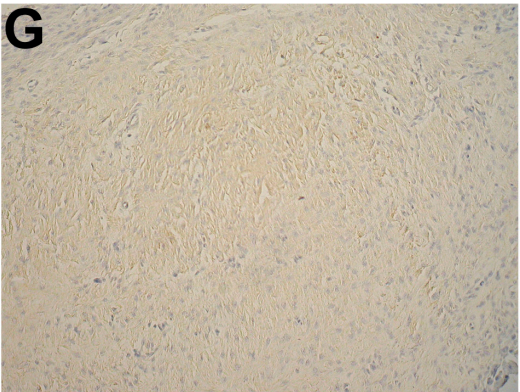
Condyle, Type I Collagen



Condyle, Type II Collagen



Disc, Type I Collagen

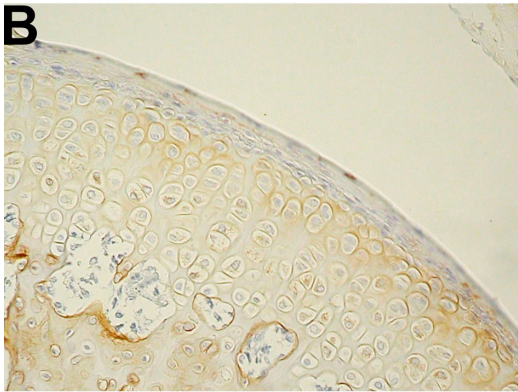


Disc, Type II Collagen

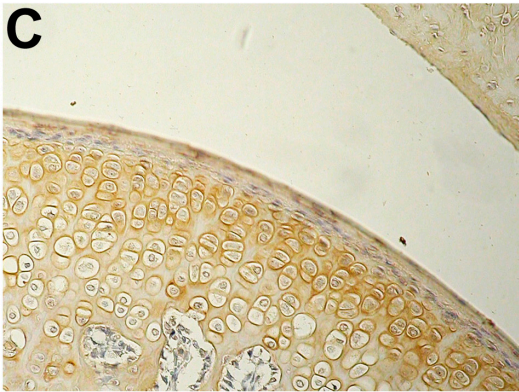
Figure 4. Dissectomized TMJ with implantation in 1 month. (A) HE analysis of the temporomandibular joint (TMJ) with a 1-month reconstituted collagen template, 320x. Cartilage exhibited mild chondroblast proliferation with minimal erosion. The disc shows fibrous degeneration. (B) Immunohistochemistry of type I collagen in a 1-month treated TMJ, 320x. (C) Immunohistochemistry of type II collagen in a 1-month treated condyle, 320x (D) Immunohistochemistry of type I collagen in 1-month treated TMJ cartilage, 1600x. Type I collagen was slightly expressed on the cartilage surface. (E) Immunohistochemistry of type II collagen in 1-month treated TMJ cartilage, 1600x. Mild type II collagen expression surrounds proliferating cells on the surface. (F) Immunohistochemistry of type I collagen in a 1-month treated TMJ disc, 1600x. Type I collagen showed normal expression. (G) Immunohistochemistry of type II collagen in a 1-month treated TMJ disc, 1600x. No significant change was observed.



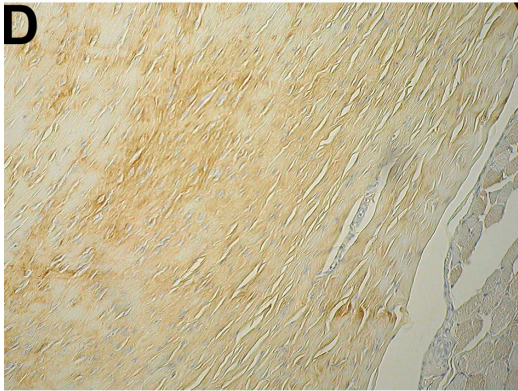
TMJ, H&E



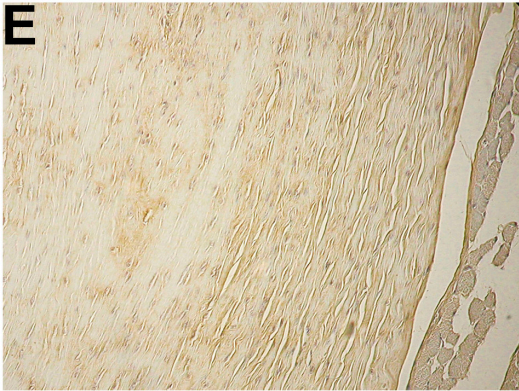
Condyle, Type I Collagen



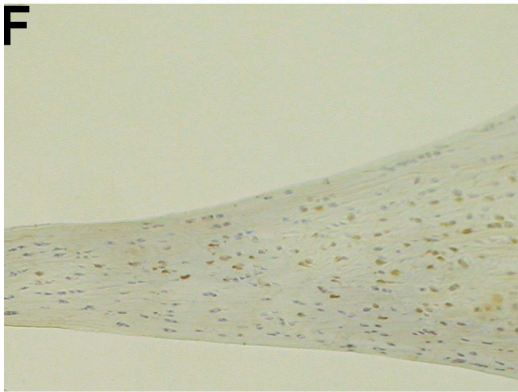
Condyle, Type II Collagen



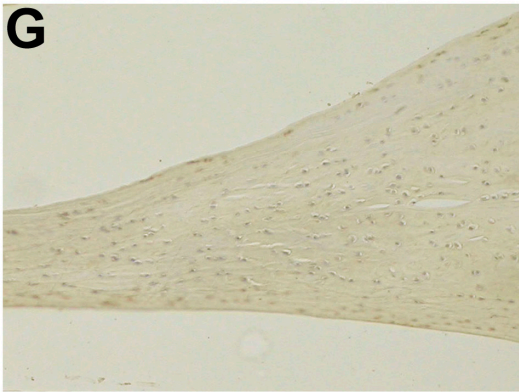
Disc, Type I Collagen



Disc, Type II Collagen



Disc, Type I Collagen



Disc, Type II Collagen

Figure 5. Dissectomized TMJ with implantation in 2 months. (A) HE stain, 320x. The disc appears to be somewhat thicker than that of the 1-month group. No erosion is present on either the tympanic fossa or condyle. (B) Immunohistochemistry of type I collagen on 2-month treated TMJ cartilage, 1600x. Type I collagen had recovered to a normal state. (C) Immunohistochemistry of type II collagen in 2-month treated TMJ cartilage, 1600x. Expression of type II collagen was normal. (D) Immunohistochemistry of type I collagen in a 2-month treated TMJ disc, 1600x. Type I collagen was expressed on wavy fibers. (E) Immunohistochemistry of type II collagen in a 2-month treated TMJ disc, 1600x. Type II collagen was expressed surrounding chondrocyte-like cells. (F) Immunohistochemistry of type I collagen in a 2-month treated TMJ disc tip, 1600x. Type I collagen was expressed in chondrocyte-like cells. (G) Immunohistochemistry of type II collagen in a 2-month treated TMJ disc tip, 1600x. Nearly normal but less type II collagen was observed.

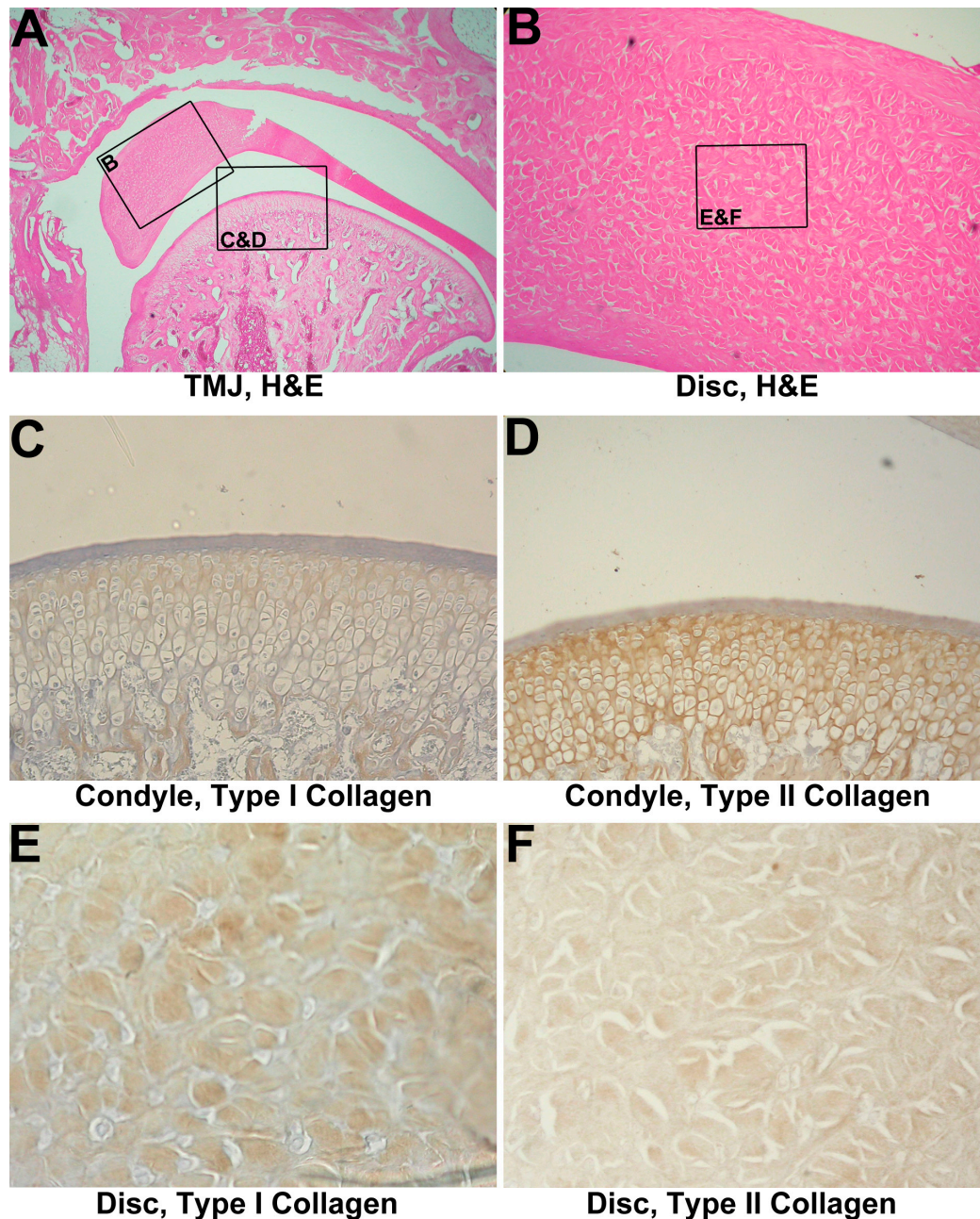


Figure 6. Discectomized TMJ with implantation in 3 months. (A) HE analysis of the temporomandibular joint (TMJ) with a 3-month reconstituted collagen template, 320x. New collagen bundles appeared in the disc and had adhered to the tympanic fossa. The condyle appears normal with a smooth condylar surface (B) HE analysis of the TMJ disc with a 3-month reconstituted collagen template, 1600x. The direction

and arrangement of new fibers differed from those of old fibers. (C)

Immunohistochemistry of type I collagen in 3-month treated TMJ cartilage, 1600x.

Type I collagen had recovered to a normal state. (D) Immunohistochemistry of type II

collagen in 3-month treated TMJ cartilage, 1600x. Expression of type II collagen was

normal. (E) Immunohistochemistry of type I collagen in a 3-month treated TMJ disc,

3200x. Type I collagen was expressed in fibers. (F) Immunohistochemistry of type II

collagen in a 3-month treated TMJ disc, 3200x. Type II collagen was expressed

randomly in fibers adjacent to cells.