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Original Article

Application of microfibrillar collagen hemostat sponge for cartilage engineering

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Abstract: The imperfections of scaffold materials have been hindering the development of cartilage tissue engineering. Avitene™ UltraFoam™ sponge, known as an active absorbable microfibrillar collagen hemostat (MCH) is an FDA approved medical material for *in vivo* application. Its safety and efficacy has been clinically tested over 30 years. This study aimed to investigate the application of the MCH sponge in cartilage engineering. The properties of the MCH bricks seeded with rabbit auricular chondrocyte and the feasibility of cartilage engineering was investigated via *in vitro* and *in vivo* study. Primary extracellular matrix (ECM) formation and type II collagen expression was detected in the pre-cultured constructs, and constructs implanted into nude mice for 8 weeks as well. Dry MCH sponge bricks without cell-seeding were implanted as controls. At 8 weeks post-implantation, mature cartilage blocks were harvested. The morphology of newly formed cartilage appeared typical mature cartilaginous tissue. The MCH collagen material had not been completely degraded both in the engineered cartilage tissue and control bricks. In conclusion, the microfibrillar collagen hemostat sponge might be an ideal candidate scaffold material in cartilage tissue engineering for clinical application.

Keywords: Cartilage, tissue engineering, scaffold, chondrocyte, microfibrillar collagen hemostat

Introduction

The identification of an ideal scaffold is essential to the success of cartilage engineering. The ideal scaffold should be biocompatible and able to maintain a structurally stable three-dimensional shape. The scaffold provides a substrate to which the cells can anchor. It should be able to provide a favorable physiological environment for cells and temporary mechanical integrity required to create an intricately shaped structure. The degradation rate of scaffold material should be balanced by the rate of tissue development to maintain the shape of the construct. Furthermore, it should be strong enough to provide a flexible structural support to supplement the neocartilage [1].

Over the last two decades, a number of materials have been used for cartilage tissue engineering, including synthetic and naturally derived porous and hydrogel-based polymers. However, so far, lack of ideal scaffold material has been hindering the development of cartilage tissue engineering [2].

Avitene™ UltraFoam™ sponge, the core ingredient is microfibrillar collagen hemostat (MCH), which is known as an active absorbable collagen hemostat, proven to accelerate clot formation. It can effectively enhance platelet aggregation and the release of proteins to form fibrin, resulting in hemostasis. The MCH is an FDA approved medical material for *in vivo* application. It's often applied for controlling bleeding in all surgical applications. Its safety and efficacy has been clinically tested over 30 years [3-9]. The Avitene™ UltraFoam™ MCH sponge is a natural derived material, without antigenicity. The ultrastructure appears as a porous struc-

ture with high porosity. It's insoluble in water, and appears not expansion after the absorption liquid. Complete degradation *in vivo* costs about 90 days. All the characteristics indicate that the Avitene™ UltraFoam™ sponge might be an ideal scaffold material for cartilage tissue engineering. This study aimed to investigate the application of the MCH sponge in cartilage engineering.

Materials and methods

An adult New Zealand white rabbit, aged 8 months, weighing 3.8 Kg, was used for auricular cartilage harvest and primary chondrocytes isolation. Six female nude mice, aged 8 weeks weighing 19-21 g, were used for *in vivo* implantation of the cultured constructs. This study was approved by the Ethics Committee of the 306th Hospital of PLA. All procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals of the 306th Hospital of PLA.

Chondrocyte culture and cell seeding

Half of the right ear of the rabbit was resected under general anaesthesia with an intramuscular injection of Sumianxin II 0.1 ml/kg (Huamu Animal Health Product Co. Ltd, Jilin Province, China). The harvested rabbit ear tissue was processed for primary chondrocytes isolation. Under sterile conditions, the skin and perichondrium were completely removed. A full-thickness piece of cartilage measuring 1 cm×1 cm was set aside for controls. The remainder cartilage was minced into 1 mm^3 fragments and digested with 0.1% collagenase type II (Invitrogen Corporation, Grand Island, NY, USA) in a D-MEM/F-12 medium (Gibco, Grand island, NY, USA) with 1% antibiotic/antimycotic solution (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China), 292 mg/mL L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) at a density of 10,000 cells/cm^2 and cultured with D-MEM/F-12 medium supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, Zhejiang Province, China), 100 U/mL penicillin and 100 mg/mL streptomycin (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China), 292 mg/mL L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mM nonessential amino acids (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China), and 50 mg/mL L-ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA). Upon reaching confluency, cell passaging was performed using routine method. The third-passage chondrocytes were used for the following study.

Ten small bricks with irregular three dimensional shape, about 3-5 mm in each dimension were cut from the Avitene™ UltraFoam™ MCH sponge (Davol Inc., Warwick, RI, USA) after the sterilized package was open. Five million chondrocytes were seeded onto each MCH brick in a 6-well culture plate. A 100 μL cell suspension, including 2.5 million cells in culture medium, was carefully pipetted onto the top of the MCH scaffold and allowed to absorb into the porous scaffold. The constructs were put into the 37°C incubator for 30 minutes, after which the constructs were flipped over and another 100 μL cell suspension was pipetted onto the other side. Again, the constructs were put into the 37°C incubator, and were flipped over for 6 times at each 30 minutes, which allowed the chondrocytes infiltrate and attach to the scaffold before submerged in culture medium.

In vitro culture

The constructs were cultured for one week at 37°C in a humidified incubator with 5% CO_2 before implantation. The culture medium was changed every other day.

Specimen implantation and harvest

After the seeded constructs were cultured *in vitro* for one week, 2 constructs were collected for histological and immunohistochemical evaluation; the other 8 constructs were subcutaneously implanted into BALB/c nude mice (Vital River Laboratories, Beijing, China), 2 constructs for each mouse. Simultaneously, 4 dry MCH sponge bricks without cells were also implanted as unseeded controls. The nude mice were sacrificed after 8 weeks, and the implants were harvested and carefully dissected from the surrounding tissue. The samples were grossly evaluated and then processed for histological and immunohistochemical examination.

Histological analysis

Specimens were processed in 10% neutral-buffered formalin for longer than 24 hours, dehydrated with gradient ethanol, embedded in paraffin, and sectioned at 5 μm. Hematoxylin

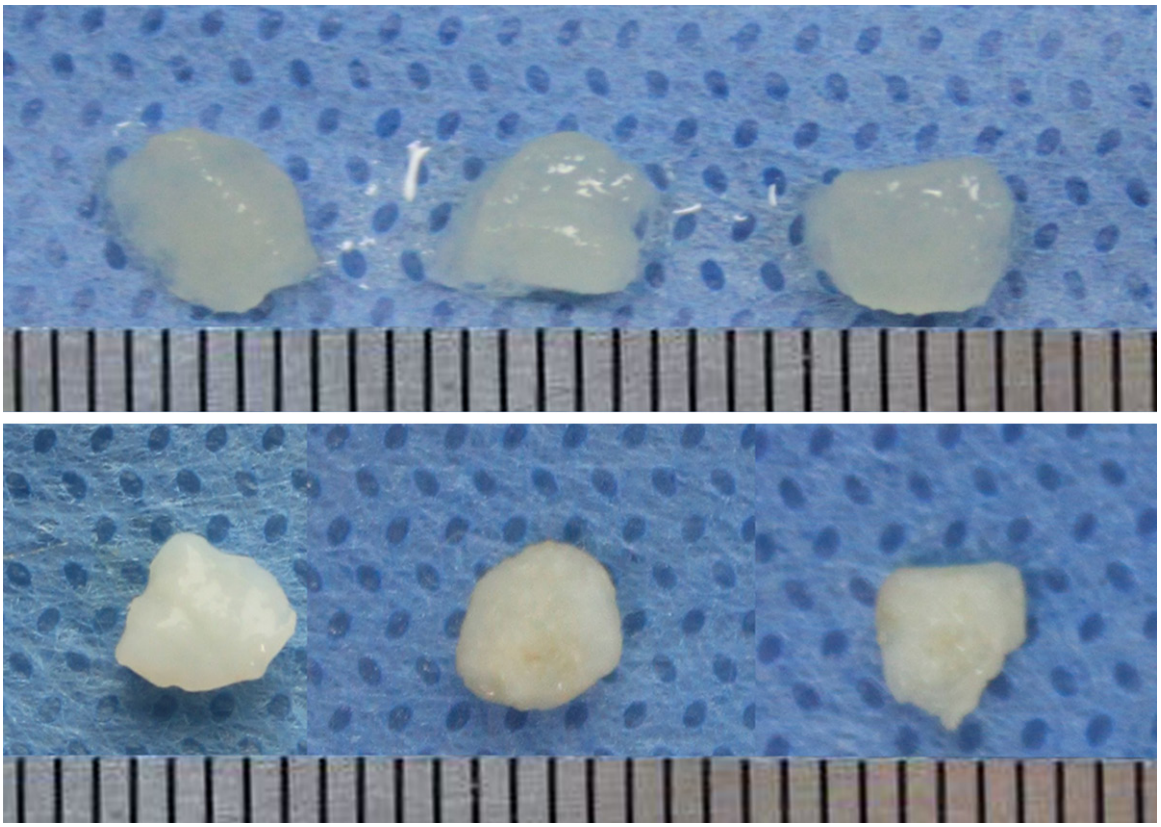

Figure 1. Gross view of the samples before implantation (upper row) and after 8-week implantation in nude mice (lower row).

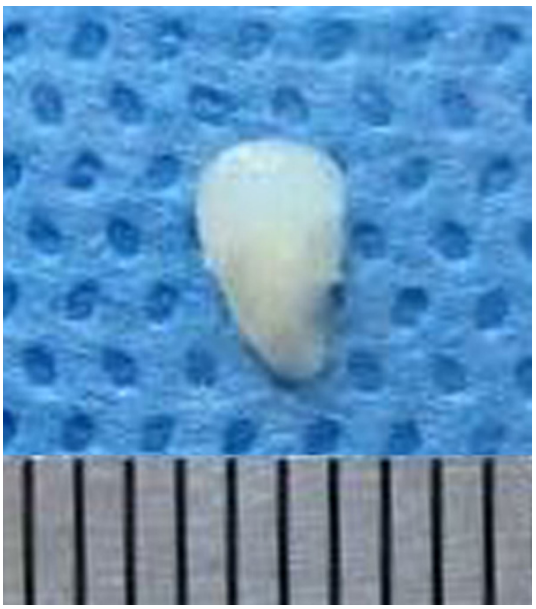

Figure 2. Gross view of the sample of control MCH scaffold after 8-week implantation in nude mouse.

and eosin (H&E), safranin-O and toluidine blue staining were performed.

Immunohistochemical analysis

Sections were heated at 60°C for 45 minutes, and then deparaffinized in sequential xylene and rehydrated in graded ethanol baths, followed by antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) for 20 minutes at 95-100°C and background signal block using 5% goat serum for 30 minutes at room temperature. Sections were incubated with rabbit polyclonal anti-Collagen II antibody (ab34712, Abcam, Cambridge, MA) for 45 minutes at room temperature, and horseradish peroxidase conjugated goat anti-rabbit IgG (Boster Biological Technologies, Wuhan, Hubei Province, China) for 30 minutes at room temperature. Diaminobenzidine substrate solution (ZSGB Biological Technologies, Beijing, China) was used for revealing the color of the antigen-antibody complexes, and hematoxylin for counterstaining.

Results

The MCH appeared moderate compressibility and absorbability. During the cell seeding, the scaffold maintained its dimensions. No shrink-

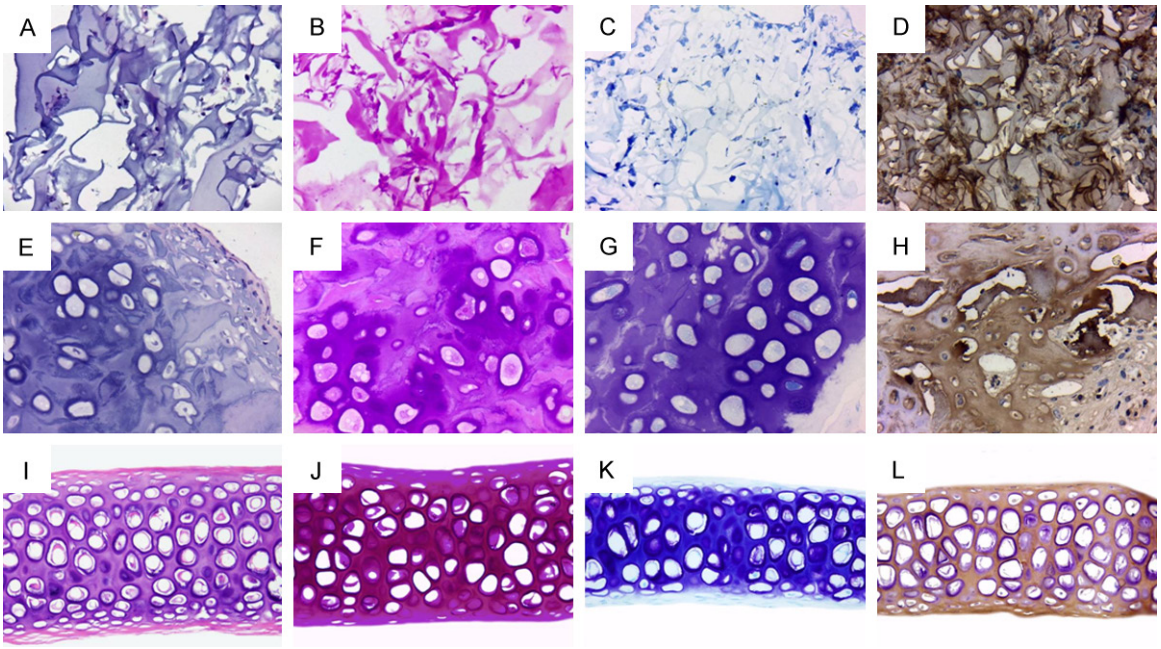

Figure 3. Histology and immunohistochemical examination of the engineered cartilage (A-H) and native rabbit auricular cartilage (I-L). (A-D) Microfibrillar collagen hemostat sponge seeded with chondrocytes and in vitro cultured for 1 week; (E-H) Neocartilage formation after 8 weeks implantation in nude mouse. (A, E and I) HE staining; (B, F and J) Safranin O staining; (C, G and K) Toluidine blue staining; (D, H and L) Immunohistochemical staining of type II collagen. Original magnification: ×400.

age was observed when adding the cell suspension. There was no considerable change in dimensions during the one-week in vitro culture.

Gross evaluation

At 8 weeks post-implantation, mature cartilage blocks were harvested. All constructs were surrounded by a thin, fibrous capsule that could be easily removed. Grossly, the tissue resembled mature cartilage, appeared white translucent, and were flexible. No apparent change in size was observed (**Figure 1**).

After the 8-week implantation, the control MCH sponge bricks had not been completely degraded. The remnant MCH material appeared white soft gel-like mass. The volumes were much less than the dry sponge bricks before implantation (**Figure 2**).

Histological and immunohistochemical evaluation

Histological evaluation revealed that the scaffold material appeared negative staining in the Toluidine blue staining (**Figure 3C**), and was stained pink in the Safranin O staining (**Figure 3B**). IHC staining revealed that the scaffold material could react with the type II collagen antibody in this study, which suggested that the MCH scaffold material comprised of type II collagen (**Figure 3D**).

It was observed that chondrocytes were dispersed throughout the scaffolds which were pre-cultured for 1 week. The chondrocytes adhered to the scaffold matrix. No obvious cartilaginous extracellular matrix formation was observed (**Figure 3A-D**).

The constructs harvested from nude mice at 8 weeks appeared typical mature cartilaginous tissue. Chondrocytes were located in isolated cartilage lacunas surrounded by matrix. The lacunas were unevenly distributed in the tissue. No vascular in-growth was observed (**Figure 3E-H**). Deposition of GAG in the extracellular matrix was evident by safranin-O (**Figure 3F**) and toluidine blue staining (**Figure 3G**). More intensive GAG deposition was observed surrounding the cartilage lacunas, similar to the cartilage capsules in native hyaline cartilage (**Figure 3I-L**).

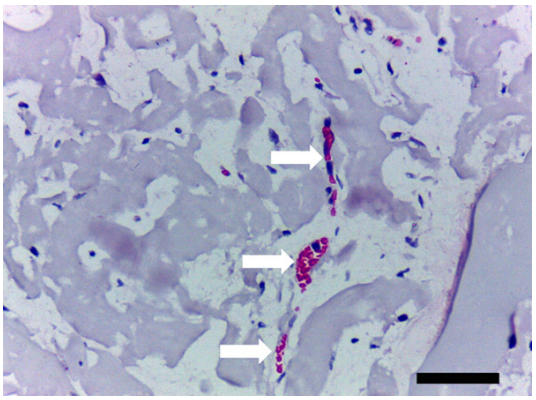

Figure 4. Histology examination of the implanted blank MCH scaffold, which showed that the microfibrillar collagen has not been degraded by 8 weeks. Sparse mouse cells in-growth and vascular in-growth were observed. Original magnification: ×400. Scale bar, 50 μm.

The MCH fiber, which had not been degraded, was distinguishable within the newly formed cartilage matrix (**Figure 3E-H**). IHC staining found abundant type II collagen formation in the engineered cartilage. Additionally, remnant MCH fiber was found positive stained for type II collagen (**Figure 3H**).

In the control scaffolds, histological evaluation found that the microfibrillar collagen has not been degraded by 8 weeks. Sparse mouse cells grew into the condensed collagen fibers. Vascular in-growth was observed (**Figure 4**).

Discussions

Avitene™ MCH is recognized as an active collagen hemostat, and has been proven to accelerate clot formation. The microfibrillar collagen accelerates clot formation by enhancing platelet aggregation and the release of proteins to form fibrin, resulting in hemostasis. MCH has been well-documented as a topical hemostatic agent in many surgical situations since it was approved for clinical use in 1976 [3-9]. The biocompatibility and biosafety has been sufficiently verified by the widespread clinical uses. The Avitene™ Ultrafoam™ sponge is made from lyophilized Avitene™ flour and water, and has a similar microfibrillar structure with Avitene™ flour. The microfibrillar structure could provide an increased surface area for cell adhesion. The histological results from this study demonstrated that the MCH scaffold provided a favorable environment for new cartilage formation.

Although the Avitene MCH is insoluble in water, it is absorbable when retained in the body after applied for stopping bleeding in the body cavity. It's recognized that the microfibrillar collagen degraded completely in 3 months in animal studies. This is the first time to adapt the MCH as the scaffold material for cartilage tissue engineering. We found that the MCH fiber had not been completely degraded after 8 weeks implantation in nude mice, both in the engineered neocartilage and in the control blank MCH scaffolds. The eventual turnover of the MCH scaffold in cartilage tissue engineering is still unknown. As we found in this study, type II collagen is a main component of the MCH. We presume that the MCH material may completely degrade or become a part of the neomatrix during neomatrix formation and remodeling. In the future, longer term studies are warranted to demonstrate the possible turnovers.

People have had high hopes on engineered auricular cartilage as a substitute for auricular reconstruction that behaves more like native ear cartilage. For more than two decades, numerous *in vitro* and *in vivo* studies have demonstrated that cartilage can be engineered using chondrocytes or mesenchymal stem cells and a variety of synthetic or naturally derived scaffold materials [2, 10-12]. In 1997, Cao et al. [13] reported a human ear-shaped construct grown on the back of a nude mouse, which was engineered using bovine articular chondrocytes and a polyglycolic acid-polylactic acid (PGA-PLA) scaffold. In the subsequent years, many researchers have sought to engineer an ideal ear structure that behaves more like native ear cartilage. However, progress stalled since the degradable scaffold could not maintain the prefabricated ear shape over longer times, even in immunodeficient animal models. Previous studies have shown that various scaffolds seeded with chondrocytes decrease in size and mass after *in vivo* implantation, which was considered as a significant shortcoming for auricular reconstruction [14, 15]. In this study, during the *in vitro* culturing and implantation in nude mice, the seeded MCH scaffold was able to maintain the shape and form neo-cartilage. The engineered neo-cartilage appeared similar to native cartilage morphologically and histologically. The result suggests that the MCH shows great promise in breaking the bottlenecks and may be an ideal scaffold material for cartilage tissue engineering.

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Disclosure of conflict of interest

None.

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