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The support of matrix accumulation and the promotion of sheep articular cartilage defects repair *in vivo* by chitosan hydrogels

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Summary

Objective: Chitosan has been widely used as an injectable scaffold in cartilage tissue engineering due to its characteristic biocompatibility and biodegradability. In this study, chitosan was used in its hydrogel form as a scaffold for chondrocytes that act to reconstruct tissue-engineered cartilage and repair articular cartilage defects in the sheep model. This study aims to find a novel way to apply chitosan in cartilage tissue engineering.

Methods: Temperature-responsive chitosan hydrogels were prepared by combining chitosan, β-sodium glycerophosphate (GP) and hydroxyethyl cellulose (HEC). Tissue-engineered cartilage reconstructions were made *in vitro* by mixing sheep chondrocytes with a chitosan hydrogel. Cell survival and matrix accumulation were analyzed after 3 weeks in culture. To collect data for *in vivo* repair, reconstructions cultured for 1 day were transplanted to the freshly prepared defects of the articular cartilage of sheep. Then at both 12 and 24 weeks after transplantation, the grafts were extracted and analyzed histologically and immunohistochemically.

Results: The results showed that the chondrocytes in the reconstructed cartilage survived and retained their ability to secrete matrix when cultured *in vitro*. Transplanted *in vivo*, the reconstructions repaired cartilage defects completely within 24 weeks. The implantation of chitosan hydrogels without chondrocytes also helps to repair cartilage defects.

Conclusions: The chitosan-based hydrogel could support matrix accumulation of chondrocytes and could repair sheep cartilage defects in 24 weeks. This study showcased the success of a new technique in its ability to repair articular cartilage defects.
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Key words: Tissue engineering, Chitosan hydrogel, Articular cartilage, Repair.

Nonstandard abbreviations: GP, β-Sodium glycerophosphate; HEC, Hydroxyethyl cellulose; AO/PI, Acridine orange/propidium iodide; H.E., Hematoxylin and eosin.

Introduction

Articular cartilage damage occurs frequently as a result of sport-related injury, disease, trauma and tumor[1]. In adult animals, human beings included, injured cartilage tissue is unable to heal spontaneously. Failure to treat damaged tissue may lead to osteoarthritis, pain and malfunction[2]. Although a variety of procedures are employed to repair cartilage damages, current methods of treatment remain unsatisfactory and inefficient, with chondroectomy[3], drilling[4], cartilage scraping[5], arthroplasty[6], transplantation of autogenic or allogenic chondrocytes[7,8], periosteum[9] as well as cartilage and bone flap being the most commonly applied[10]. Repairing large defects of articular cartilage continues to be a daunting challenge for clinical surgeons[11].

With the development of tissue engineering, reconstructing cartilage provides a new method of cartilage repair. The main method employed to engineer cartilage involves the use of seeding cells and scaffolds. Scaffolds are used because they provide a three dimensional environment highly desirable for the production of cartilaginous tissues[12]. Both synthetic[13–15] and natural[16–18] scaffolding materials have been used for cell delivery in cartilage regeneration.

Many reports exist that detail successful repairs of cartilage defects with the use of different synthetic polymers. However, these materials are flawed with examples being some of the following reasons: (1) their use involving the creation and accumulation of acidic by-products; (2) their low biocompatibility; and (3) the possible toxic by-products of these materials' degradation after implantation, which may elicit an inflammatory response[19].

In contrast, natural materials often have high levels of biocompatibility, since their interactions with cells *via* cell surface receptors lessen chances for implantation rejection,

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and these materials also are easily biodegraded[12]. Natural materials that have been explored as bioactive scaffolds for cartilage engineering include chitosan[20,21], collagen[22], atelocollagen gel[23,24], fibrin[25], alginate[26], and agarose[27]*et al.* Satisfactory results were obtained for all. Ochi *et al.* showed that implanting chondrocytes with the use of an atelocollagen gel could promote restoration of the articular cartilage of the knee[23].

Chitosan is a natural renewable resource. It is composed of glucosamine and *N*-acetylglucosamine monomers and has many attractive properties including biocompatibility, biodegradability, non-toxicity, physiological inertness, remarkable affinity to proteins, bacterial resistance, and haemostaticity. It also improves wound-healing activity in the connective tissues of several species[28]. Furthermore, chitosan shares some characteristics with articular cartilage, e.g., various glycosaminoglycan (GAGs) and hyaluronic acid[29], making it an ideal scaffolding material in articular cartilage engineering. Hoemann *et al.*[30] reconstructed tissue-engineered cartilage *in vitro* for transplantation into a nude mouse by using injectable temperature-responsive chitosan as a scaffold. They also injected the chitosan alone into rabbit articular cartilage to repair defects. Optimal results were obtained after at least 1 week *in vivo*. Chervier *et al.*[31] showed improved cartilage repairs upon the application of chitosan-GP/blood implants to the marrow-stimulated cartilage lesions. These studies suggested that chitosan hydrogel was a desirable scaffold to use to repair cartilage defects. However, since the injected chitosan needs at least 10 min to gel after transplantation, it may flow out of the joint cavity, resulting in the formation of cartilage-like tissue ectopically. This can cause complications like pain, therefore, injection may not be the best way to deliver chitosan to articular cartilage defects[32]. Ito *et al.* repaired osteochondral defects in the rabbit model with a tissue-engineered chondral plug made of atelocollagen sponge and Poly-L-Lactide-Acid mesh. The chondral plug could be adjusted to the proper shape and orientation to fit the intrinsic defect[24]. This kind of "*in situ* forming" approach could help solve the aforementioned problems that pertain to using chitosan as a scaffold. In this study, we have reconstructed tissue-engineered cartilage *in vitro* by using temperature-responsive chitosan hydrogels as scaffolds before transplantation *in vivo*. The reconstructions could avoid liquid chitosan runoff into the articular cavity, and could be easily modified to adapt to the shape of the defects[12]. We used autologous chondrocytes as the seeding cells and sheep as the experimental animals. No report on the same topic has been discovered by the author yet.

Methods

ANIMALS AND GROUPS

Twenty four skeletally mature and healthy sheep (male or female, 8–12 months old, 20–25 kg mean body weight) were provided by the Experimental Animals Center of the Beijing Institute of Basic Medical Sciences, and were kept in a controlled environment with access to food and water. All experiments were approved by the Animal Experimental Committee of the Beijing Institute of Basic Medical Sciences.

The sheep were randomly divided into three groups. In the experimental group ($n = 12$), the sheep were implanted with the tissue-engineered cartilage. In control group 1 ($n = 6$), the sheep were implanted with chitosan hydrogel. The sheep with the untreated defects served as control group 2 ($n = 6$).

ISOLATION OF CHONDROCYTES

In the experimental group, all animals were anesthetized through intramuscular administration of ketamine (0.01 mg/kg). The articular cartilage tissue was cut off from the left knee joint of the non-weight-bearing areas of the sheep, and then minced into pieces of about 1 mm^3. The minced cartilage tissue was transferred to a glass spinner flask containing H-DMEM (Invitrogen, Carlsbad, Calif) with 25 mM HEPES (Sigma, St. Louis, MO), 1.5 mg/ml collagenase II (Sigma, St. Louis, MO) and spun on a magnetic stirrer at 37°C for 8–12 h. The obtained cell suspension was filtered through a 120 μm pore size nylon mesh filter and washed twice with phosphate-buffered saline (PBS). The chondrocytes were harvested by centrifugation at 1500 rpm for 5 min. Cell number and viability were determined by means of a hemocytometer and trypan blue vital dye. The chondrocytes were cultured in H-DMEM and 10% fetal bovine serum (Hyclone, Logan, UTA) in an incubator at 37°C, 5% CO_2. All manipulations were performed under sterile conditions. We collected chondrocytes of passage three to serve as seeding cells.

FORMULATION OF CHITOSAN HYDROGEL

The temperature-responsive chitosan hydrogel was prepared according to Hoemann CD *et al.*[30] Chitosan was obtained from Pronova (Oslo, Norway, Product No UP CL 113 degree of deacetylation (DDA): 86%; 12%HCl; apparent viscosity: 16 mPa.s; dry matter content: 96.0%; Endotoxins: 230EU/gram). Protasan UP113 powder was dissolved in the distilled water at 2%(w/v) chitosan, then autoclave sterilized. The chitosan solutions were mixed with concentrated stock solutions of filter-sterilized β-sodium glycerophosphate (GP, Sigma, 11.5%w/v, molecular weight: 216.04), to yield a liquid chitosan-GP solution with 1.6% chitosan, 108 mM GP. The final pH is 6.8. Chitosan hydrogels were formed by mixing 10 ml chitosan-GP solution with 2.5 ml cross-linking solution consisting of 2.5% (w/v in H-DMEM) filter-sterilized hydroxyethyl cellulose (HEC) (Fluka, St. Louis, MO, viscosity: 1500 mPa.s). They were incubated at 37°C for 10–15 min.

RECONSTRUCTION OF TISSUE-ENGINEERED CARTILAGE *IN VITRO*

About 4×10^7 chondrocytes were mixed with 1 ml chitosan hydrogel and poured into a 4-well cell culture plate, and then incubated at 37°C for 10–15 min to allow it to gel. We constructed 15 pieces of tissue-engineered cartilage altogether at one time. After 1 day of *in vitro* culture, 12 pieces were transplanted to the defects *in vivo* to aid repairs. Cell viability in the hydrogel was assessed by using acridine orange-propidium iodide (AO/PI) staining. Briefly, the stock solution (AO: 670 μmol/L, PI: 750 μmol/L) was prepared with Dulbeccos solution and kept in the dark at 4°C. Just before use, 0.01 ml AO and 1.0 ml PI were mixed, diluted by 10 times with Dulbeccos solution, and then passed through a 0.22 μm filter membrane. The hydrogels containing chondrocytes were incubated with the AO/PI mixture for 10 min and observed under a fluorescence microscope. Live cells were stained in green (AO) whereas dead cells were colored red (PI). To find the accumulation of matrix in the tissue-engineered cartilage, the remaining three pieces of reconstructed tissue were cultured *in vitro* for 3 weeks and analyzed by using hematoxylin and eosin (H.E.), toluidine blue, safranin O and type II collagen immunohistochemical staining.

BIOMECHANICAL PROPERTIES OF RECONSTRUCTED CARTILAGE *IN VITRO*

Biomechanical properties of tissue-engineered cartilage were assessed *via* stress-relaxation tests in uniaxial, unconfined compression using the Mach-1™ micromechanical testing system (BioSyntech, Laval, QC) according to Hoemann CD *et al.*[30]. For reconstructed cartilage cultured *in vitro*, two successive compression ramps each with an amplitude corresponding to 10% of the sample thickness were applied at a strain rate of 1%/s. Equilibrium was estimated when the load changed by less than 0.05 g/min. Stress-relaxation curves from the second ramp (10–20% strain) of reconstructed cartilage were fit to a fibril reinforced poroelastic model to obtain the compressive equilibrium modulus (E_m) and tensile fibril modulus (E_f).

AUTOLOGOUS TRANSPLANTATION OF TISSUE-ENGINEERED CARTILAGE

All animals were anesthetized through intramuscular administration of ketamine (0.01 mg/kg). The right rear limb was shaved and then disinfected. A 4 cm-long incision was made over the right knee of each animal. The patella was dislocated laterally. The knee was flexed 90° to expose the non-weight-bearing area of the medial femoral condyle. One defect, 8 mm in diameter and 3 mm in depth, was created mechanically with a hollow trephine (external diameter, 8 mm). The depth of penetration was limited to 3 mm by a line marked on the hollow trephine. In the experimental group, the defects were filled with the corresponding construction of tissue-engineered cartilage. In control group 1, the defects were filled only with the chitosan hydrogel. In

control group 2, the defects were kept empty and closed without implantation. The joint cyst was carefully closed by precise suturing. The soft tissue flap was sutured to its original site. All animals received an injection of 160,000 units of penicillin for 3 days and allowed free movement post-operation. At 12 and then 24 weeks after implantation, half of the total number of sheep was killed each time by intravenous injection of a lethal dose of barbiturate. The knee joints were removed for further study.

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL EVALUATION

The harvested samples were first examined grossly and photographed, and then were sawn apart through the center of the defects into several slices − cartilage defect, adjacent cartilage, and underlying bone. Most of the slices were immersed in 4% paraformaldehyde buffer (pH 7.4) for histological examination. The rest with cartilage defects were frozen in liquid nitrogen for biochemical analysis.

The dissected samples perpendicular to the surface of the implantation sites were dehydrated through successive concentrations of alcohol (70% up to absolute). The blocks were cleared in xylene before being embedded in paraffin and then were cut with a microtome (RM2165 rotary microtome; Leica Microsystems, Nussloch, Germany). The samples were sectioned to 5 μm and stained with H.E., toluidine blue, safranin O/fast green and type II collagen immunohistochemical stains.

For immunohistochemistry, the sections were washed in PBS, digested with 0.1% trypsin for 30 min at 37°C, and then pretreated with 5% goat serum (GS) for 20 min at room temperature to block nonspecific reactions. Polyclonal antibodies to type II collagen (Maixin, Fujian, China) were incubated with the sections for 60 min. The sections were rinsed in PBS and incubated with post-blocking (Maixin, Fujian, China) for 20 min. They were then rinsed three times with PBS, treated for 30 min with poly-HRP Anti-Ms/Rb IgG (Maixin, Fujian, China), rinsed three times with PBS, and treated for 5–10 min with 0.02% diaminobenzidine in 0.01 M Tris buffer (pH 7.6) containing 0.005% H_2O_2.

The specimens were graded semiquantitatively on the basis of the predominant nature of the repaired tissue, matrix staining, regularity of the surface structural integrity, thickness of the repair, apposition between the repaired cartilage and the surrounding normal cartilage, freedom from degenerative changes in the repair tissue, and freedom from the surrounding normal cartilage as described by O' Driscoll *et al.*[34] Differences in histological scoring between groups were analyzed by ANOVA. Statistical analyses were performed with SPSS 11.0. Significance was accepted at $P < 0.01$. Results are reported as mean ± standard deviation.

BIOCHEMICAL ANALYSIS

Frozen samples were carefully crushed and digested with papain (0.5 mg/ml; Sigma) at 65°C for 4 h. A 50 μl sample of digested tissue was mixed with 2.5 ml of Alcian blue (Sigma, St. Louis, MO). The samples were analyzed with a spectrophotometer (DU640; Beckman Coulter, Fullerton, CA) at an optical wavelength of 480 nm. The amount of GAG present in the harvested tissue was determined at 480 nm by comparing it with a chondroitin sulfate (Sigma, St. Louis, MO) standard curve[33]. Statistical analysis was performed with the SPSS 11.0. Data are shown as mean ± standard deviation. A *t*-test was performed to analyze the different amounts of GAG between the experimental group and the control groups. Differences at $P < 0.01$ were considered significant.

Results

FORMULATION OF CHITOSAN HYDROGEL AND RECONSTRUCTION OF TISSUE-ENGINEERED CARTILAGE *IN VITRO*

The temperature-responsive chitosan hydrogels were kept in liquid [Fig. 1(A)] at room temperature and then solidified [Fig. 1(B)] at 37°C for 10–15 min. The size of the reconstructed cartilage approximates that of the well of the 4-well plate [Fig. 1(C)]. The AO/PI staining showed that the chondrocytes remained >90% viable in the chitosan matrix after being cultured for 1 day *in vitro* [Fig. 1(D)].

ACCUMULATION OF MATRIX IN THE TISSUE-ENGINEERED CARTILAGE

The results showed that the chondrocytes in the chitosan hydrogels accumulated pericellular sulfated GAG-containing matrix. H.E. staining showed that the chondrocytes in the chitosan hydrogels were normal in appearance [Fig. 2(A)]. Moreover, in the chitosan hydrogel, we also observed type II collagen immunohistochemical staining activity [Fig. 2(B)]. The toluidine blue staining and safranin O staining indicated that chondrocytes could produce the cartilage matrix in the chitosan hydrogels [Fig. 2(C, D)].

BIOMECHANICAL PROPERTIES OF RECONSTRUCTED CARTILAGE *IN VITRO*

The biomechanical properties of the reconstructed cartilage after 0 days and after 1 day of *in vitro* culture were assessed. The results showed that although the mechanical properties of the reconstructed cartilage increased after 1 day of culture compared with that of 0 day of culture (Table I), the mechanical properties (E_m and E_f) of reconstructed cartilage remained significantly inferior to native tissue (the data were previously reported by Hoemann CD *et al.*[30]).

GROSS OBSERVATION OF THE ARTICULAR CARTILAGE REPAIR 24 WEEKS POST-OPERATION

All animals survived the follow-up period of 12 or 24 weeks without signs of major wound infection, limited range of motion, or synovitis in the operated knees. No free tissue-engineered cartilage was found outside of the cartilage defects in the joint cavity. At 12 weeks post-operation, most of the defects treated with tissue-engineered cartilage in the experimental group were repaired, leaving a relatively consistent and smooth joint surface. At 24 weeks post-operation, the defected part of the cartilage in the experimental group was covered by the smooth, consistent, glistening white hyaline tissue almost indistinguishable from the surrounding normal cartilage. No clear signs of margin with normal cartilage could be spotted on the surface of the regenerated areas [Fig. 3(A)]. In contrast, the defects in control group 1 were partially repaired with fiber-like tissue, leaving a small depression in the defect areas [Fig. 3(B)]. And the defects in control group 2 detected a thin and irregular surface tissue with obvious defects and cracks surrounding the normal cartilage [Fig. 3(C)].

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL EVALUATION OF THE ARTICULAR CARTILAGE REPAIR

In the experimental group, H.E. staining displayed the boundary of original and renewed cartilage 12 weeks post-operation. The cells in the renewed cartilage were somewhat different from those in the normal cartilage tissue [Fig. 4(A)]. The formation of cartilage-like tissue could be seen through toluidine blue staining and safranin O staining, indicating a considerable amount of proteoglycan [Fig. 4(B,C)]. In control group 1, the defect areas were filled with the remaining chitosan surrounded by fibrocartilage tissues [Fig. 4(D)]. It seemed that no cartilage-like tissue was formed [Fig. 4(E, F)]. In control group 2, H.E. staining showed that there were no typical cells [Fig. 4(G)], the negative toluidine blue staining indicated that no cartilage tissue existed [Fig. 4(H)], but a small quantity of fibrous tissues could be seen along the margin of defects [Fig. 4(I)].

At 24 weeks post-operation, H.E. staining showed that the newly-formed cartilage had matured. The cells of the new cartilage were similar to those of normal chondrocytes [Fig. 5(A)]. The regenerated hyaline-like cartilage had less intensive toluidine blue staining than adjacent normal cartilage [Fig. 5(B)]. The typical structure of hyaline cartilage

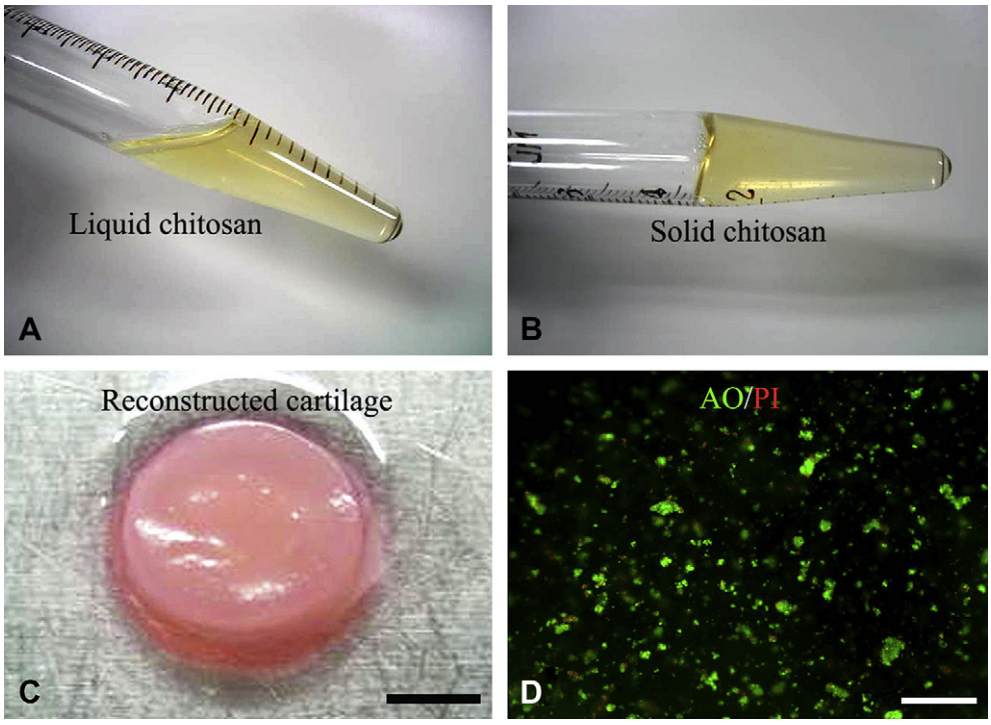

Fig. 1. Formulation of chitosan hydrogel and reconstruction of tissue-engineered cartilage *in vitro*. A, In room temperature, the chitosan hydrogels were keep in liquid; B, The chitosan hydrogels kept at 37°C in 10−15 min were solidified; C, The reconstructed cartilage in the 4-well plate; D, The AO/PI staining showed that the chondrocytes remained >90% viable in the solid chitosan matrix after cultured 1 day *in vitro*, AO: green; PI: red. Bar = 0.5 cm(C); 100 μm (D).

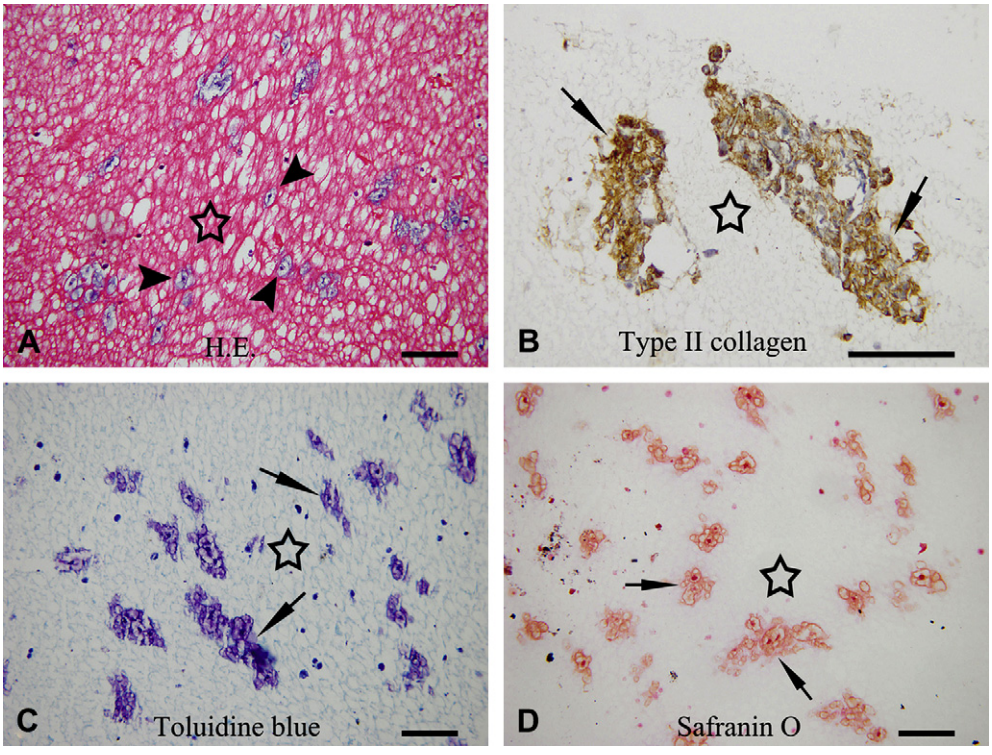

Fig. 2. Accumulation of matrix in the tissue-engineered cartilage. Histology and immunohistochemistry of chondrocytes cultured in chitosan hydrogels 3 weeks. The results showed that the chondrocytes in the chitosan hydrogels accumulated pericellular sulfated GAG-containing matrix. A, H.E. staining; B, Type II collagen immunohistochemical staining; C, Toluidine blue staining; D, Safranin O staining. Star: chitosan hydrogel; Arrowhead: cell nucleus; Arrow: matrix of the chondrocytes. Bar = 100 μm.

Table I
Mechanical properties of reconstructed cartilage in vitro

Days cultured	Reconstructed cartilage	
	0 day ($n=4$)	1day ($n=4$)
E_m (equilibrium modulus, kPa)	1.5 ± 0.2	5.1 ± 0.6
E_f (fibril modulus, kPa)	40 ± 16	110 ± 9

lacunae was apparent in the regenerated area [Fig. 5(C)]. The regenerated surface was demonstrated to be hyaline-like by type II collagen immunohistochemical staining [Fig. 5(D)]. In control group 1, no chitosan hydrogels were observed [Fig. 5(E)]. The defects were partly filled with some renewed tissues when modestly stained with toluidine blue and safranin O/fast green. The renewed tissue was proved to be cartilage [Fig. 5(F, G)], yet type II collagen immunohistochemical staining showed the cartilage to be fibrocartilage other than hyaline-like cartilage [Fig. 5(H). In control group 2, the defect areas remained empty, with H.E. staining indicating that no cells existed in the defect areas [Fig. 5(I)]. The negative staining by toluidine blue, safranin O/fast green showed that the cartilage defects contained only loose fibrous tissues [Fig. 5(J, K)]. So did Type II collagen immunohistochemical staining [Fig. 5(L)].

BIOCHEMICAL ANALYSIS

GAG content is shown in Table II and Table III. GAG was at 76% and 85% of the content for normal cartilage in tissue-engineered cartilage at 12 and 24 weeks post-operation respectively. In contrast, such content in the other two groups was much lower. Statistical analysis demonstrated a significant difference between the experimental group and the two control groups ($P < 0.01$) at both 12 and 24 weeks post-operation. A slight difference was also noted between the experimental group and normal cartilage ($P < 0.05$).

STATISTICAL ANALYSIS

The mean histological scores were 17.5 ± 1.87 (12 weeks post-transplantation) and 22.2 ± 1.17 (24 weeks post-transplantation) in the experimental group, 3.5 ± 1.87 (12 weeks post-transplantation) and 13.5 ± 1.38 (24 weeks post-transplantation) in control group 1, and 2.17 ± 1.20 (12 weeks post-transplantation) and 10.5 ± 1.87 (24 weeks post-transplantation) in control group 2 (Table IV). The scores of the experimental group were significantly different when compared with control groups 1 and 2 ($P < 0.01$). These results indicated that the tissue-engineered cartilage formed with chitosan hydrogel had desirable defect-repairing capabilities.

Discussion

Cartilage tissue engineering provided a novel and ideal method for the repair of cartilage defects[12], marking breakthroughs in the areas of stem cell treatment, biomaterials, and biomechanics. The utilization of new materials for tissue-engineered cartilage scaffolds is a priority of scientific research[28]. In this study, we reconstructed tissue-engineered cartilage by using temperature-responsive chitosan hydrogels as scaffolds, and after a short-term *in vitro* culture, transplanted the reconstructed tissue into big animal models to repair articular cartilage defects. In just 24 weeks after transplantation, the cartilage defects were repaired completely. The results indicated that the tissue-engineered cartilage using temperature-responsive chitosan hydrogel as a scaffold evidently possessed the ability to repair cartilage defects.

The preparation method and choice of scaffold material were key to the development of these tissue-engineered constructs. The scaffolds provided a 3-dimensional environment for the chondrocytes' *in vitro* and *in vivo* cultures. They could also support the matrix production of chondrocytes[35]. Over the past few years, hydrogel materials, especially natural hydrogel materials, have become more and more attractive for use in tissue engineering. There are some kinds of hydrogels used specifically for cartilage tissue engineering, such as chitosan hydrogels[36], alginate hydrogels[37], polymer oligo(poly(ethylene glycol) fumarate) (OPF) hydrogels[38], and poly(ethylene glycol)-based (PEGDA) photopolymerizing hydrogels[39]. Among these scaffold materials, chitosan may be one of the better

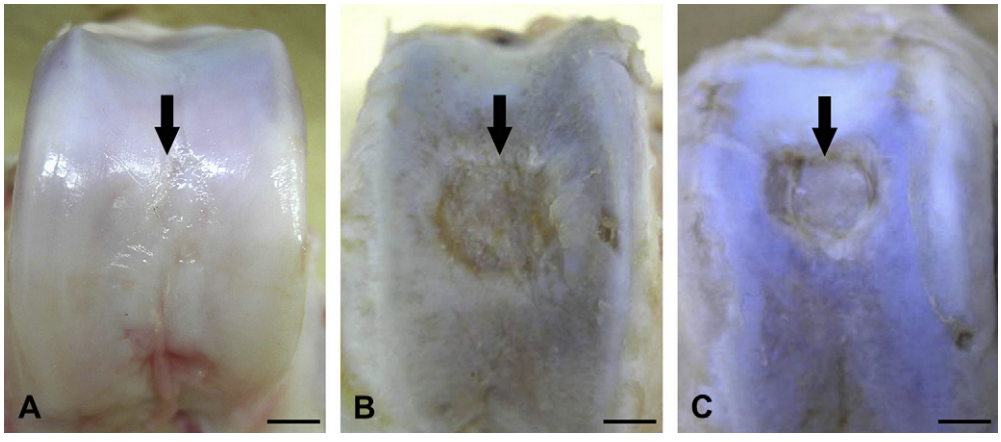

Fig. 3. Gross observation of the articular cartilage repair at 24 weeks post-operation. A, the defect part of the cartilage in the experimental group was covered by the smooth, consistent, glistening white hyaline tissue nearly indistinguishable from the surrounding normal cartilage. No clear signs of margin with normal cartilage could be spotted on the surface of the regenerated areas; B, The defects in control group 1 were partially repaired with fiber-like tissue, leaving a small depression in the defect areas; C, The defects in control group 2 detected a thin and irregular surface tissue, with obvious defects and cracks surrounding the normal cartilage. Arrow: the defect; Bar = 0.5 cm.

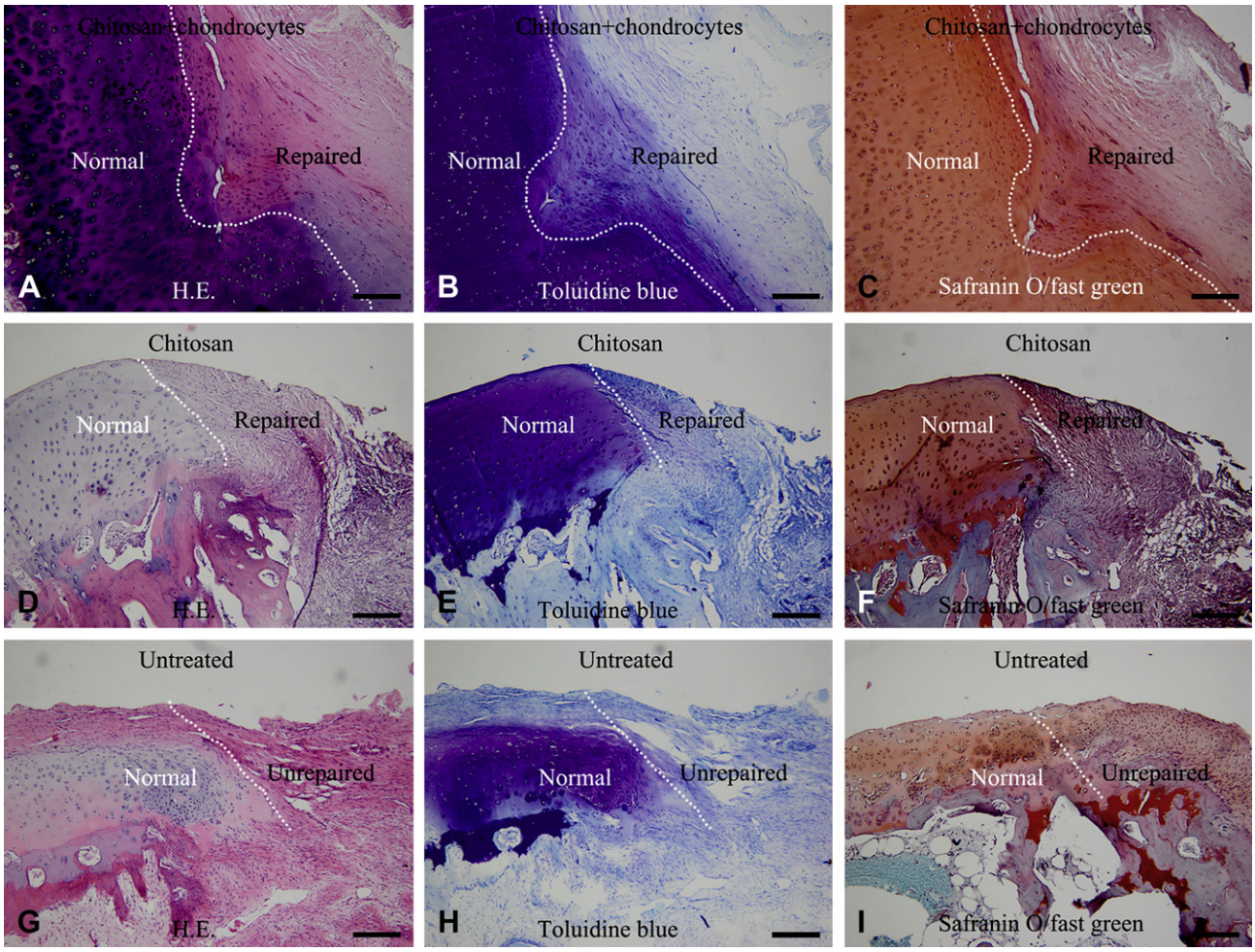

Fig. 4. Histological evaluation of the articular cartilage repair at 12 weeks post-operation. (A–C), In experimental group, H.E. staining displayed that the boundary of normal cartilage and renewed cartilage. The cells in the renewed cartilage were somewhat different from those in the normal cartilage tissue (A); The formation of cartilage-like tissue could be seen with toluidine blue (B) staining and safranin O staining (C), indicating a considerable amount of proteoglycan; (D–F), In control group 1, the defect areas were filled with the remaining chitosan hydrogels materials surrounded by fibrocartilage tissues (D). It seemed that no cartilage-like tissue was formed as shown by toluidine blue (E) staining and safranin O staining (F); (G–I), In control group 2, H.E. staining showed that there were no typical cells (G), the negatively toluidine blue staining indicated that no cartilage tissue (H), but only a small quantity of fibrous tissues could be seen along the margin of defects (I). Dotted line indicated the boundary of the normal cartilage and the renewed cartilage. Bar = 100 μm.

choices due to its desirable biocompatibility and biodegradability. The chitosan hydrogel has two types: temperature-responsive and UV-radiation-responsive[40]. Reports show that temperature-responsive chitosan hydrogels were able to repair cartilage lesions[30,31], therefore we used temperature-responsive chitosan hydrogels as the chondrocytes' scaffold to reconstruct tissue-engineered cartilage *in vitro*. Transplantation of these reconstructions to repair the cartilage defects of big animals proved successful. At both 12 and 24 weeks, the results showed that the chitosan hydrogel could enhance ability for repairs in adult cartilage. Nevertheless, the results were even better when the hydrogel contained chondrocytes. At 24 weeks post-operation, the defected part of the cartilage in the experimental group was completely repaired with hyaline cartilage tissue. H.E. staining showed that the newly-formed cartilage had matured [Fig. 5(A)]. The regenerated hyaline-like cartilage had less intensive toluidine blue staining than adjacent normal cartilage [Fig. 5(B)]. The typical structure of hyaline cartilage lacunae was apparent in the regenerated area [Fig. 5(C)]. The regenerated surface was demonstrated to be hyaline-like by type II collagen immunohistochemical staining [Fig. 5(D)].These results demonstrated that chitosan hydrogels are applicable to cartilage tissue engineering.

Biocompatibility of the chitosan hydrogel greatly impacts the repairs of defects in the regenerated tissue-engineered cartilage[41]. The viscosity, DDA and content of endotoxic products in chitosan affects the biocompatibility of the hydrogel. The results from the 3 week long *in vitro* chitosan hydrogel with autologous chondrocytes co-culture were satisfactory, since the chondrocytes in chitosan hydrogel retained normal growth and the ability to secrete certain matrix. H.E. staining showed that the chondrocytes in the chitosan hydrogels were normal in appearance [Fig. 2(A)]. In the chitosan hydrogel, we also observed type II collagen immunohistochemical staining activity [Fig. 2(B)]. The toluidine blue staining and safranin O staining indicated that chondrocytes could produce the cartilage matrix in the chitosan hydrogels [Fig. 2(C, D)]. These results indicated that

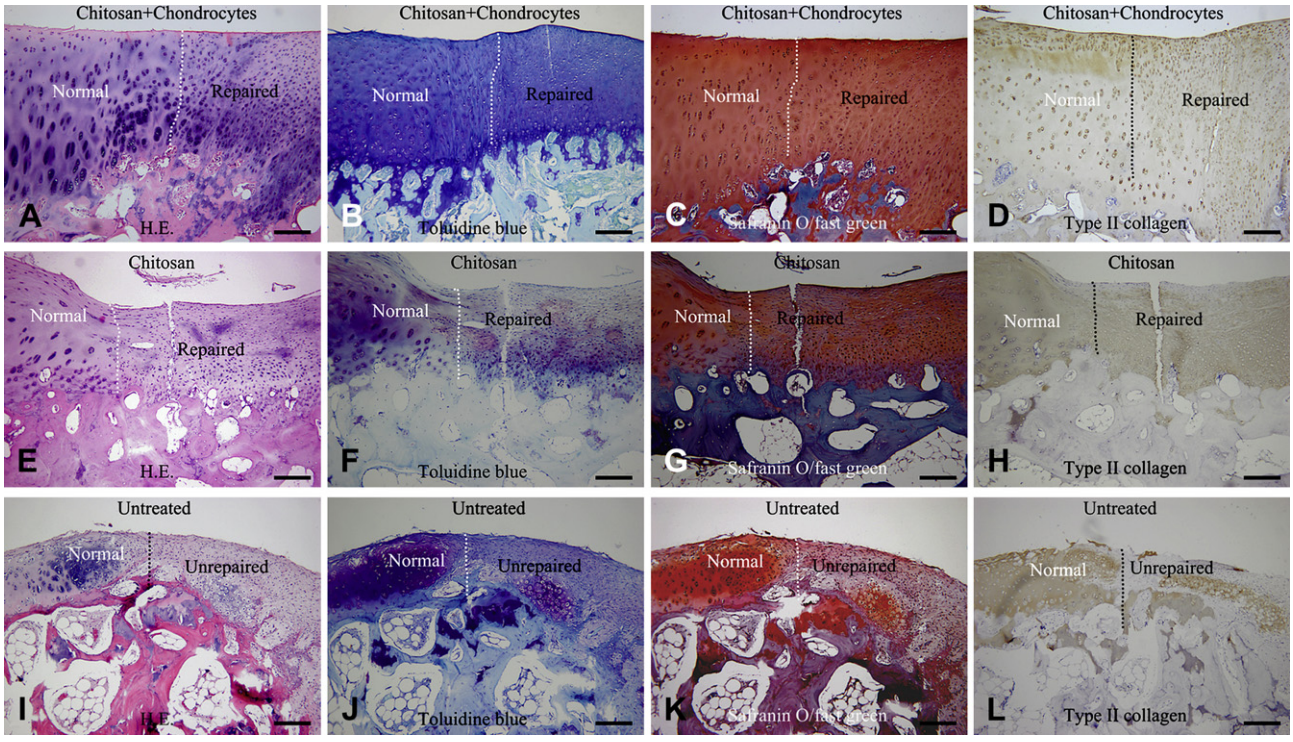

Fig. 5. Histological and immunohistochemical evaluation of the articular cartilage repair at 24 weeks post-operation. (A–D), In experimental group, H.E. staining showed that newly-formed cartilage became more mature. The cells in the newly cartilage were similar to the normal chondrocytes (A). The regenerated hyaline-like cartilage had less intensive toluidine blue staining than adjacent normal cartilage (B). A typical structure of hyaline cartilage lacunae is apparent in the regenerated area (C). The regenerated surface was demonstrated to be hyaline-like by type II collagen immunohistochemical staining (D); (E–H), In control group 1, no chitosan hydrogels were observed (E). The defects were partly filled with some renewed tissues when modestly stained with toluidine blue (F) and safranin O/fast green (G). Type II collagen immunohistochemical staining showed the cartilage to be fibrocartilage other than hyaline-like cartilage (H); (I–L), In control group 2, H.E. staining indicated that the defect areas have no cells existence (I). The negative staining by toluidine blue (J), safranin O/fast green (K) showed that the cartilage defects containing only some loose fibrous tissues. Type II collagen immunohistochemical staining contained the same (L). Dotted line indicated the boundary of the normal cartilage and the renewed cartilage. Bar = 100 μm.

chitosan hydrogel has desirable biocompatibility. It could provide a comfortable 3-dimensional environment for the seeded chondrocytes.

The mechanical intensity of the scaffold is one of the factors that affects the repair of cartilage defects with use of reconstructed cartilage. It was reported that the mechanical intensity may influence the defect-repairing ability of tissue-engineered cartilage to some extent[42]. Hoemann *et al.* assessed the biomechanical properties of reconstructed cartilage *via* stress-relaxation tests in uniaxial, unconfined compression *in vitro* and *in vivo*, with the results showing that mechanical properties of these *in vitro* and *in vivo* constructs remain significantly inferior to those of native tissue, but this did not affect its persistence in osteochondral defects for at least 1 week *in vivo*[30]. In this study, the tissue-engineered cartilage was reconstructed using the same method as in Hoemann's study. The reconstructed cartilage was transplanted 1 day after culture, so the biomechanical properties of the reconstructed cartilage after 0 days and after 1 day of *in vitro* culture were assessed. The results showed that the mechanical properties of the reconstructed cartilage also remained significantly inferior to native tissue.

Table II
Quantification of GAGs 12 weeks post-transplantation

Joint	Normal cartilage	Experimental group	Control group 1	Control group 2
1	138	112	12	15
2	146	106	12	9
3	140	105	18	11
4	142	110	16	10
5	141	102	15	8
6	144	115	16	11
Mean	141.83 ± 2.86	108.33 ± 4.84*	14.83 ± 2.40†	10.67 ± 2.42†

*Differences ($P < 0.05$) between the experimental group and normal cartilage were revealed as analyzed by *t* test.

†Significant differences ($P < 0.01$) between the experimental group and either control group 1 or control group 2 were revealed as analyzed by *t* test.

Table III
Quantification of GAGs 24 weeks post-transplantation

Joint	Normal cartilage	Experimental group	Control group 1	Control group 2
1	142	125	48	28
2	145	123	47	34
3	141	114	51	33
4	136	120	50	24
5	147	118	45	28
6	139	120	44	27
Mean	141.67 ± 3.98	120.00 ± 3.85*	47.50 ± 2.74†	29.00 ± 3.79†

*Different ($P < 0.05$) between the experimental group and normal cartilage were revealed as analyzed by *t* test.
†Significantly different ($P < 0.01$) between the experimental group and either control group 1 or control group 2 were revealed as analyzed by *t* test.

Table IV
Histological scores of experimental and control groups

Number	Experimental group		Control group 1		Control group 2	
	12 weeks	24 weeks	12 weeks	24 weeks	12 weeks	24 weeks
1	18	22	2	13	3	8
2	16	22	1	12	1	11
3	19	21	4	12	2	13
4	15	23	6	15	4	12
5	20	21	3	15	2	9
6	17	24	5	14	1	10
Mean	17.5 ± 1.87*	22.2 ± 1.17*	3.5 ± 1.87	13.5 ± 1.38	2.17 ± 1.17	10.5 ± 1.87

*Significantly different ($P < 0.01$) between the experimental group and either control group 1 or control group 2 were revealed as analyzed by analysis of variance (ANOVA).

In addition, the results from the autologous chondrocyte implantation (ACI), a study on the injection of scaffold-free chondrocytes, which is already approbated by the Food and Drug Administration (FDA)[43], were available with up to a 16-year follow-up examination. More than 80% of the patients have shown improvement with relatively few complications[9]. Furthermore, the scaffold of matrix-autologous chondrocyte implantation (MACI) was I/III collagen membrane[44] that had insufficient mechanical intensity, yet the results from MACI were satisfactory. So we suggested that the mechanical intensity of the scaffolds was not the only factor important in tissue-engineered cartilage repairs. Nevertheless, we still expect to see experiments in future studies that are able to increase the mechanical intensity of reconstructed cartilage.

In the field of cartilage repair or replacement, tissue engineering may hold much influence in the future[9]. Many new materials and medical technologies are being developed to aid the regeneration of healthy tissues after cartilage damage[45]. In this study, tissue-engineered cartilage *via* combining a novel, natural scaffold technology with autologous chondrocytes proved to have great potential for regenerating damaged knee cartilage in the animal model *in vivo*. However, the widespread clinical efficacy of this treatment modality continues to be hampered by several problems, such as the mechanical intensity strength of the scaffolds, cell proliferation ability and implantation difficulties[46]. Thus, in view of these challenges, future research is anticipated.

Conflict of interest

None.

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