Original Article
Influence of biological scaffold regulation on the proliferation of chondrocytes and the repair of articular cartilage

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Abstract: Purpose: To investigate the effects of hard tissue engineering scaffold (the material is β-TCP) with different micro-structures on the proliferation of chondrocytes, and the influence of its composite erythrocytes on the repair of articular cartilage defects. Methods: Rabbit cartilage cells were on β-TCP bioceramic scaffold with different microstructures in vitro, the proliferation growth trend of chondrocytes within the scaffold was calculated, and a optimal micro-structure suitable for cartilage cell growth was determined. Composite chondrocytes were implanted into rabbit models of articular cartilage defects, and the repair situation was observed. Results: the bioceramic scaffold with an inner diameter of 120 μm and an aperture of 500-630 μm was suitable for the growth of cartilage cells. Scaffold loaded with second generation of cartilage cell suspension got a top histological score of 20.76±2.13, which was closely similar to that of normal cartilage. Conclusion: When loaded with the second generation of cartilage cells, the β-TCP biological ceramic scaffold with a pore size of 500-630 μm, and an inner diameter of 120 μm, shows a best repairing effect on animal articular cartilage defects.

Keywords: Cartilage, cartilage cell proliferation, articular cartilage repair, biological ceramic

Introduction

With the rapid development of cell biology and materials science, the application of tissue engineering techniques to repair cartilage erosion has become possible. The optimization of cartilage tissue engineering scaffold materials and seed cell source is the prerequisite and key to the application of this technology. Moreover, the selection of scaffold materials is one of the key factors in organ reconstruction [1] Ideal tissue engineering bio-materials should have the following characteristics: ① good bio compatibility, that means no significant cytotoxicity, inflammation or immune rejection; ② suitably biodegradable, that means the rate of degradation and absorption is adaptable to cell and tissue growth rates; ③ appropriate pore size, high porosity (>90%) and connected hole shape (inner connection); ④ specific three-dimensional shape in order to obtain desired tissue or organ shape; ⑤ high surface area and suitable surface physicochemical properties to facili-

tate cell adhesion, proliferation and differentiation, as well as biological signaling molecules like loaded growth factors; ⑥ matched structural strength with the mechanical properties of the tissues at implant site [2, 3]. Calcium phosphate bioceramics are nontoxic to the human body and give no immune response, so they have good bio-compatibility, bio-activity, bone conduction and even bone induction functions [4]. In addition, calcium phosphate bio-ceramics are conducive to the growth of bone tissue after being implanted in body; therefore, they are widely used in bone tissue engineering research. Wherein, β-calcium phosphate (β-TCP≥95%, Ca/P:1.50) has good biodegradability and a calcium to phosphorus ratio of 1.5 (in mass), which is close to normal bone tissue. The porous β-TCP can also provide a large surface area, which is conducive to cell proliferation, differentiation and metabolism. When implanted into a living organism, The Ca, P elements released by degradation of scaffold materials go into the circulatory system of the living body, and have certain

promoting effects on the formation of new bone [5, 6]. Therefore, β-TCP is generally considered as an ideal material for bone tissue engineering inorganic scaffold with attractive application prospects. There are studies which have confirmed that mature cartilage cells can secrete TGF-β [5], which plays an important role in the differentiation of ES cells into cartilage cells [7, 8]. Moreover, mature cartilage cells secrete extracellular matrix, such as type II collagen, which also have the function of cartilage inducing [9]. The structures with controllable aperture diameter and inner connection can better regulate the attachment and growth of cartilage cells, and determine the relationship between secretion of extra-cellular matrix and growth factors, so as to select a β-TCP bio-ceramic scaffold with suitable micro-structure, which can be used as a suitable scaffold for cartilage tissue engineering. This study investigated the possibility of β-TCP as scaffold, chondrocytes as seed cells to construct tissue engineered biological artificial cartilage, the influence of its micro-structure in the cartilage tissue engineering construction, its repairing effect on the articular cartilage defects of New Zealand white rabbits, and suitable micro-structures of β-TCP bioceramic materials for the construction of tissue engineered cartilage.

Materials and methods

Materials

Scaffold: Using β-TCP biological ceramics with controllable microstructures, a biological ceramics scaffold suitable for the repair of articular cartilage defects was designed and produced [10]. β-TCP biological ceramics with controllable microstructures was prepared using patented technology, and according to our measurement, the purity was greater than 95%, the porosity was between 60%~85%; the spherical aperture was 200~800 micron; the connected track inside the aperture was 50~400 micron; the open-cell rate was greater than 99%; the connected insides of the aperture were uniform and conntrollable, with a compressive strength greater than 3 Mpa. It was suitable for the requirements of tissue growth.

Through our research, the form of scaffold shall be a cylinder, 4 mm in diameter, 6-7 mm in depth.

Animal: Select 36 two months old healthy New Zealand white rabbits of either gender, weighing 2.0-2.5 kg. The rabbits were provided by the Laboratory Animal Center of Fudan University. Take rabbit's femoral trochlea near the intercondylar fossa as the research model of the rabbit's articular cartilage defects and the choice for the implantation site of hard β-TCP biological ceramics scaffold (β-TCP≥95%, Ca/P:1.50, Biomaterials Co. Ltd, Shanghai).

Seed cells: The costal chondrocyte of 3 month old New Zealand white rabbits, clean grade.

Instrument: Hard tissue slicing machine (Wetzlar Germany); grinding machine (Norderstedt Germany); Super clean workbench (BIO-HAZARD, VCM-420 Taiwan); CO_2 incubator (FORMA THERMO 3110 USA); Inverted phase contrast microscope (LEICA Germany); Inverted fluorescence microscope (IX71 OLYMPUS Japan); The pH (METTLER TOLEDO 320, Switzerland); -20°C refrigerator (SANYO Japan); Centrifuge (TDL-40B Shanghai); Electronic analytical balance (TOLEDO AB204-E, METTLER Shanghai); Ele ctromagnetic agitator (SM STUART 24 UK); Infrared drying box (766-2 Shanghai); High pressure steam sterilization pot (LABO MLS-320 SANYO Japan); Pure water machine (CLASSIC PALL, PURELAB USA); Enzyme linked instrument (TECANSUNRISE Japan); Blood cell count board (XB.K.25 China); 25 cm² culture dish (NUNCLON, Germany); 15 ml, 50 ml centrifuge tube (BD Company USA); Training Board (BD, USA).

Reagent: DMEM culture solution (PH7.4, GIBCO USA); FBS (GIBCO USA); Trypsin (Shanghai, China); Type II collagenase (SIGMA USA); EDTA-Na₂ (analysis of pure, Shanghai); Myllicin (1 × 105 U/L, 100 mg/L, HYCLONE); DMSO cryopreservation solution (HYCLONE USA); Thiazole blue (MTT, Beijing); Two methyl (DMSO, Beijing); Glutamine (Gibico, USA); Vitamin C (Gibico, USA); Collagen type II anti-mouse anti-rabbit (merke USA); Drop plus horseradish enzyme labeled Goat anti mouse (Dr. de Zhejiang); Sodium (animal); Sodium sulfide (Shanghai); EDTA (Beijing).

Methods

Cell inoculation, sampling: Select second generation rib cartilage cells with good morphology to prepare cell suspension with a cell count of $4×10^7$/ml. Divide the β-TCP biological ceramic

Table 1. Scoring criteria for histology of cartilage

1. Main repair tissue traits		
① Cell morphology	Cartilagines vera	4
	Incompletely differentiated mesenchymal cells	2
	Fibrous tissue or bone tissue	0
② Matrix special staining	Normal or near normal	3
	Moderate staining	2
	Slight staining	1
	Non-staining	0
2. Structure characteristics		
① Surface light degree	Smooth and complete	3
	Horizontal stratification	2
	25-100% of crack thickness	1
	Severe tissue rupture and fibrosis	0
② Tissue integrity	Normal	2
	Light is broken, including the cyst	1
	Severe degeneration	0
③ Cartilage thickness	100% Normal cartilage thickness	2
	50-100% Normal cartilage thickness	1
	0-50% Normal cartilage thickness	0
④ Integration with neighboring cartilage	Both sides are integrated well	2
	Unilateral or bilateral integration	1
	Bilateral integration is not well	0
3. Whether there is cell degeneration in Repair tissue		
① Cell volume reduction	Normal	3
	Mild	2
	Moderate	1
	Severe	0
② Chondrocyte clustering	None	2
	Less than 25%	1
	25-100%	0
③ No surrounding cartilage degeneration	Normal cartilage cell number, Normal soft color, No cluster like chondrocyte aggregation	3
	Normal cartilage cell number, middle soft color, light cluster like chondrocyte aggregation	2
	Mild or moderate number of decreased cells, slightly soft	1
	Cell number severely reduced, No staining or very light	0

scaffolds of circular disk-shape (14 mm in diameter and 4 mm in height) into six groups: 120 μm inner connection tract, 400-500 μm aperture; 120 μm inner connection tract, 500-630 μm aperture; 120 μm inner connection tract, 630-700 μm aperture; 100 μm inner connection tract, 500-630 μm aperture; 150 μm inner connection tract, 500-630 μm aperture; 200 μm inner connection tract, 500-630 μm aperture.

Cell suspension was loaded by a sterile syringe into β-TCP biological ceramics scaffold materials. Standed for several minutes, then took out the materials and put them into a 12-well culture plate, dropping 1 ml DMEM broth containing 10% FBS (GIBCO American). After 1 hour standing, dropped 10 ml DMEM (GIBCO American) broth containing 10% FBS again. Then put them into the incubator at 37°C, with saturated humidity and 5% CO_2. Changed the culture medium the next day. Put it under a microscope to observe the adhesive situation between cells and scaffold edge after 4 h, 2 d, 3 d, 5 d, and 7 d of inoculation. Culture them for 7 days, 14 days and 28 days, respectively. In case of accidental error during the research, 6 samples shall be taken in each term.

Hard tissues slicing and treatment: Gradiently dehydrated and embedded the scaffold, used a hard tissue slicing machine to slice along the cylindrical centerline, with the initial thickness of the slice being 200 μm. Make 50 μm-thick abrasive discs using a grinding miller. Used acid taste-magenta dye to go on histological staining with the hard tissueslices, and observed the distribution of the cell proliferation wthin the scaffold by microscope (LEICA
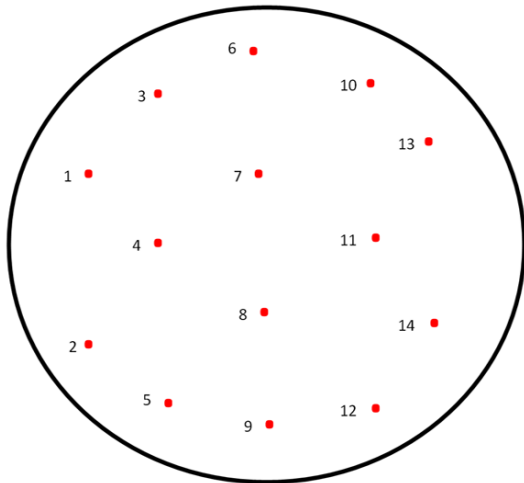
Figure 1. The distribution of the material points.

Germany). Selected 15 points in the same areas of the materials to photograph with the low power lens; observed the proliferation and attachment of the cells and calculated the number of cells.

SEM testing of the cell attachment: The scanning electron microscopic (SEM) photographs were taken at PhilipsXL30 scanning electron microscope, the operating voltage wass 20 kV. Slit the materials and observed the attachment of the cell at the surface and inside.

Type II collagen immunohistochemistry stain.

After data statistics, the biological ceramic scaffold structure most suitable for the growth of chondrocyte was determined (SPSS10.0).

Experimental animal grouping: The biological ceramics with optimized scaffold structure were divided into three groups: Blank control group (without implantation of cells in the scaffold), SPC group, that is the scaffold loaded with primary generation of rib cartilage cell suspension, and the SCC group, that is the scaffold loaded with second generation of rib cartilage cell suspension . The rib cartilage primary cells and the second generation cells were prepared into cell suspension, then let each group of cell suspension penetrated into the materials of β-TCP biological ceramic scaffold. After several minutes of standing, tissue-engineering cartilage formed.

Embedding cartilage material into the model of intercondylar cartilage defects: After anesthesia by intramuscular injection of pentobarbital sodium (0.02 mg/kg), expose the load-bearing surface of the medial condyles of femur of two hind legs of every rabbit. Drill with 4 mm drill at the femoral trochlea articular surface of the same position of condyles of two hind legs to dislodge the cartilage and the subchondral bone. Every defect was 4 mm in diameter and 7 mm in depth. Implant scaffold alone, scaffold loaded with rib cartilage primary cells suspension, or the scaffold loaded with the second generation of rib cartilage suspension, respectively. Each group had 12 rabbits (24 keens). Dissect the suture of each wound carefully, so each laboratory animal could be injected with 80000 units of penicillin for 3 days. Two groups of the rabbits were both walking weight-bearing.

Materials and processing: Took 4 rabbits (8 keens) from each group at 4 w, 12 w or 16 w after operation, and injected a pneumatic needle into the ear vein to put them to death and took a sample of bilateral femoral condyle. Then the samples were processed as follow: decalcification, toluidine blue colorimetric assay (Positive result: the mucus of the chondrocyte appears bluish violet), the "0" stain of bright green-saffron (positive result: cartilage tissue appears red) and type II collagen immunohistochemistry stain.

Histological evaluation of cartilage: Evaluate the morphology of cartilage according to the method of D'Driscoll, Keeley and Salter [11]. The standard for evaluation is shown as follow (**Table 1**).

Result

Cell counting

Fifteen points from each material were chosen (**Figure 1**) under an optical microscope to take a photo and compute the number of cells. The results are showed as: 1) cell recombination rate decreased with the increase of diameter of apertures and inner connections; 2) cell-attachment rate reduced with the increase of aperture diameters, and reached the highest value with an inner connection diameter of 120 microns; 3) cell proliferation rate decreased with the increase of aperture diameter, and reached the highest value with an aperture of 500-630 microns. The results have a direct guiding significance and application value to tissue engineering optimization structure. The results are shown in **Table 2**, with a schematic diagram as shown in **Figures 2** and **3**.

Table 2. Material cell proliferation condition (means ± sd, n=6)

	Group		1 W	2 W	3 W
	Inner connection μm	Aperture μm			
A	120	400-500	24.72±3.63	135.50±34.65	190.61±60.28
B	120	500-630	43.64±8.97	239.21±57.57	456.32±36.69
C	120	630-700	62.87±9.63	172.11±26.36	114.38±27.96
D	100	500-630	49.28±3.69	125.16±15.62	42.35±14.32
E	150	500-630	22.52±2.63	98.83±24.32	20.08±2.32
F	200	500-630	36.69±12.65	17.60±1.20	25.03±3.36
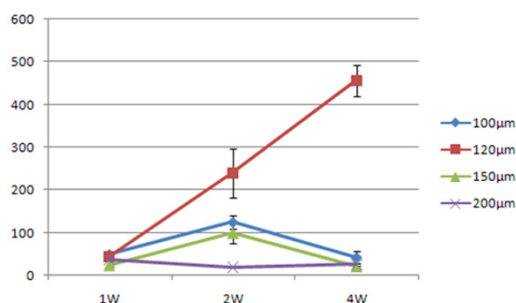
Figure 2. Cell growth of different inner diameter bracket in the same aperture of 500-630 μm.
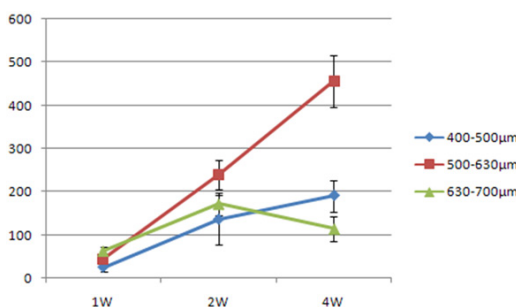

Figure 3. Cell growth of different aperture in the same inner connection diameter of 120 μm.

SEM testing of cell attachment

Cleave the materials with an inner connection diameter of 120 microns and an aperture of 500-630 microns. Observe the surface of the material and the cells inside, which revealed that the cell attachment and proliferation were in good condition. The bioceramic scafold material with the inner connection diameter of 120 microns and the aperture of 500-630 microns, was most suitable for the growth of cartilage cells (**Figure 4**).

The immunohistochemistry results: Selected scafold materials at 4 w time point, used 10%

EDTA for decalcification, and embedded the materials with wax. TypeII collagen immunohistochemical result revealed as positive, which illustrated that rib cartilage cells did not lose their phenotype under the cultivation of 3D scaffolds outside the body. As is shown in **Figure 5**.

The general observation of the condyle cartilage defect of rabbit model

One month after the operation, all three groups of cartilage hadn't recovered.

Two months after the operation, there were no obvious signs of recovery in the cartilage defects for both the blank control group and the SPC group primary. The β-TCP scaffolds showed no obvious degradation, and the boundary around the cartilage defects was clear; the cartilage defect was sunken and the texture was soft, with signs of degradation in the surrounding normal cartilage. While in the SSC group, parts of the cartilage defect recovered, and the surrounding cartilage defect boundary was clear, with sunken cartilage defect and soft texture.

Four months after the operation, in the control group, there was no obvious signs of cartilage repair in the defect area, and there was no obvious degradation of β-TCP scaffolds. The borders of cartilage defects were clear, the cartilage defect was sunken and the texture is soft, and there were signs of degradation in the surrounding normal cartilage. Four months after the operation, in the SPC group, the cartilage defect was covered with a granulation tissue or chondroid tissue. The boundary of granulation tissue was not clear, the color was dark red, and the texture was soft, but the surrounding normal cartilage had no obvious signs of degeneration. Four months after the operation, in the SSC group, the cartilage defect area was cov-
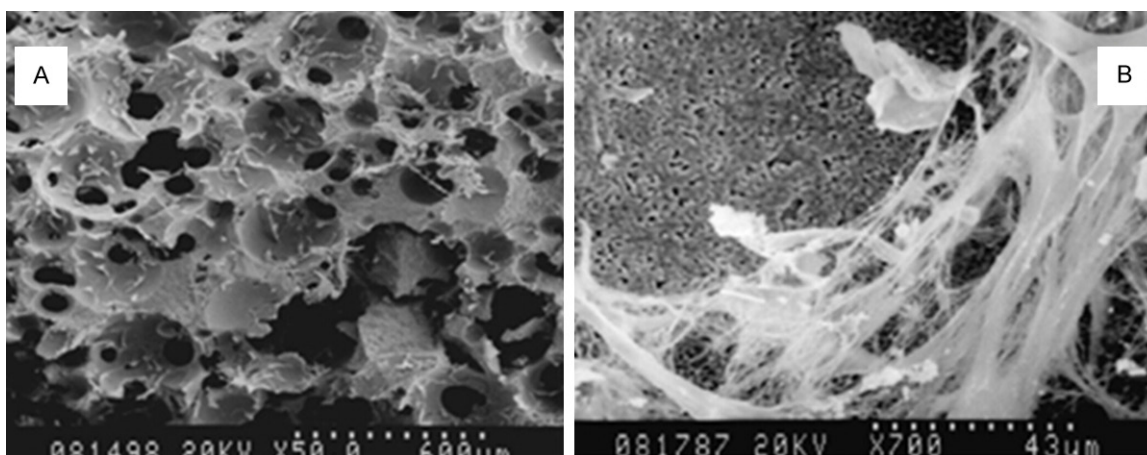
Figure 4. Cell attachment in the inner join diameter of 120 μm and aperture of 500-630 μm.
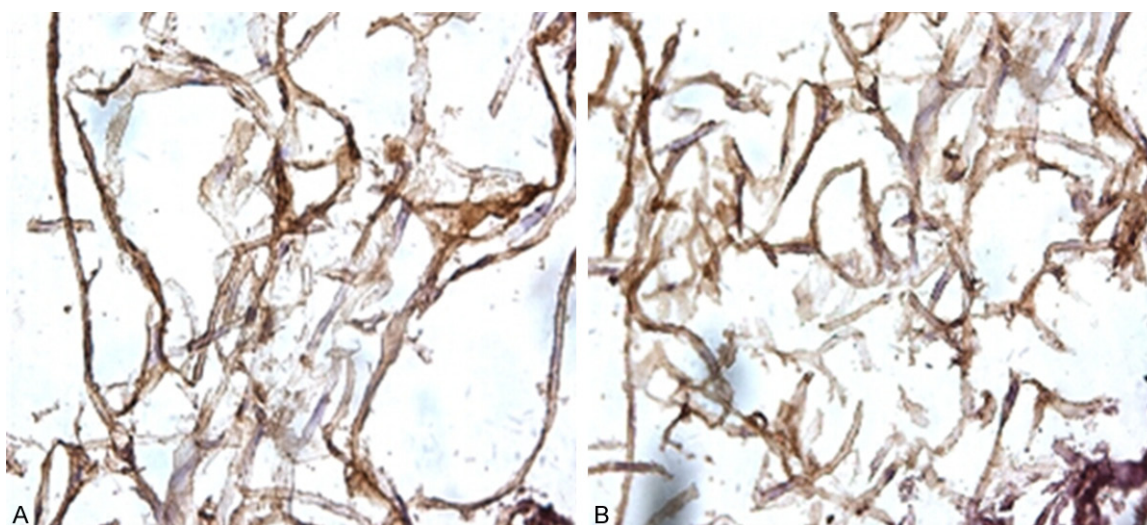

Figure 5. Case of immunohistochemistry.

ered by a new white chondroid tissue with a smooth surface. The boundary between the repaired tissue and the surrounding normal were not clear. All the repaired tissue and the normal cartilage were similar, the texture was strong, of rich elasticity, and the surrounding normal cartilage showed no obvious signs of degradation. Part of the β-TCP materials beneath the cartilage had been degraded, and part of them might be transformed to the ossification. (As shown in **Figure 6**).

Toluidine blue staining

When stained by toluene gel blue, the normal cartilage was blue, color from shallow to deep layer of cartilage gradually deepened. The color of deep cartilage and subchondral trabecular bone were consistent, and the cartilage lacuna

was negatively stained. The repaired tissues of blank control group were lightly stained, and the matrix appeared as fibrous tissue. Four months after the operation, in the SPC group, the repaired tissue was a light blue cartilage, the matrix fiber was bulky and reticulate, superficial (equivalent to a subcutaneous layer of normal cartilage) fiber was horizontal; and the middle (equivalent to a liquid layer and the middle layer of normal cartilage) fiber was unordered. Four months after the operation, in the SSC group, the staining of repaired tissue was close to normal cartilage, the matrix fiber was bulky and horizontally arranged, and similar to the subchondral bone (equivalent to normal cartilage calcification layer),hyaline cartilage could be seen in matrix, with cartilage lacuna structure. (As shown in **Figure 7**).
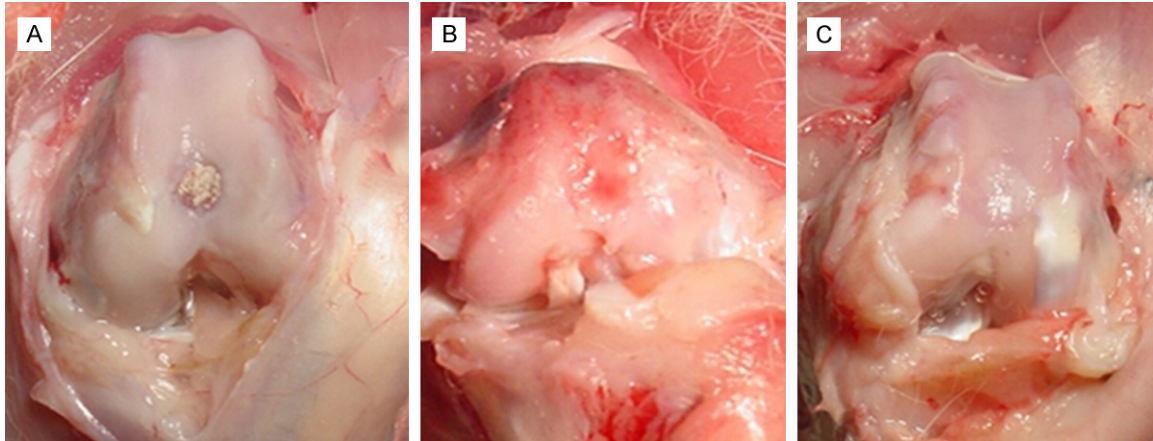
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Figure 6. General observation after surgery for 4 months. A. Blank control; B. Bracket and Primary cartilage cell suspension. C. Bracket and 2nd generation of cartilage cell suspension.

Figure 7. Toluidine blue staining after surgery for 4 months. A. Blank control; B. Bracket and Primary cartilage cell suspension. C. Bracket and 2nd generation of cartilage cell suspension.
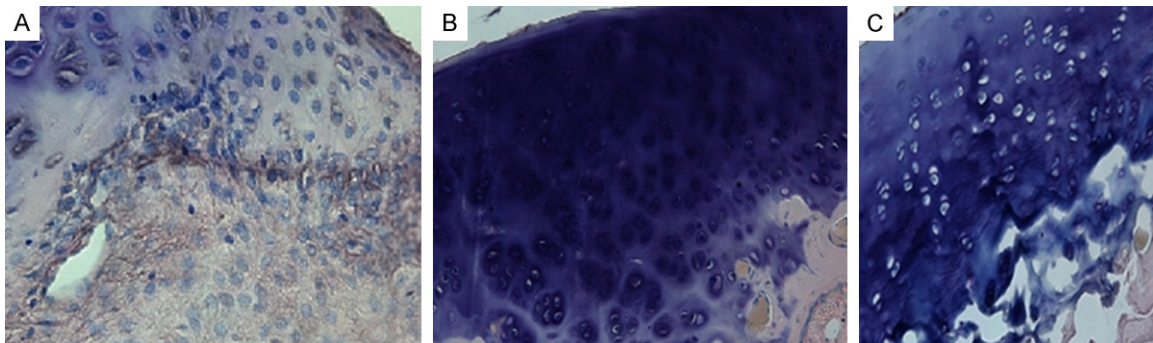
Safranin staining-O

When stained by safranin-O, the normal cartilage was red , with the color gradually deepening from the shallow, middle to deep cartilage. The repaired tissue staining of the blank control group was not obvious, with little cartilage lacuna structure. Four months after the operation, the safranin staining-O of the repaired tissues of the SPC group group were lightly stained; the cartilage structure was visible in both the middle and deep layers, clear in the deep layer. Four months after the operation, the staining of the SSC group s group was deeper; the structure of the cartilage lacunae was visible in all the shallow, middle and deep layers, clear in the middle and deep layers; the middle lacunae was arranged disorderly, but in the deep layer, they were arranged in columns. (As shown in **Figure 8**).

Collagen type II immunohistochemical staining

Four months after the operation of the SSC group, the type II collagen immunohistochemical staining of the scaffold material and new cartilage tissues were tan in color (**Figure 9**).

Cartilage histological grading

Normal cartilage score was 24 points (full score). The higher was score of a repaired organization form, the closer it got to the normal cartilage. The score was higher in turn among the blank control group, the SPC group and the SSC group group, which was shown in detail in **Table 3**.

Discussion

Because of its advantage in maintaining cell phenotype, three-dimensional cultures have
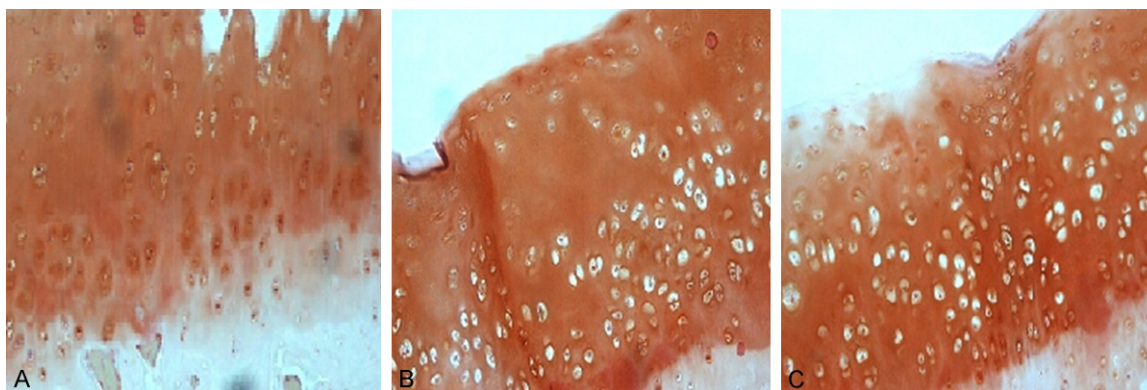
Figure 8. Fan red-O staining after surgery for 4 months. A. Blank control; B. Bracket and Primary cartilage cell suspension. C. Bracket and 2nd generation of cartilage cell suspension
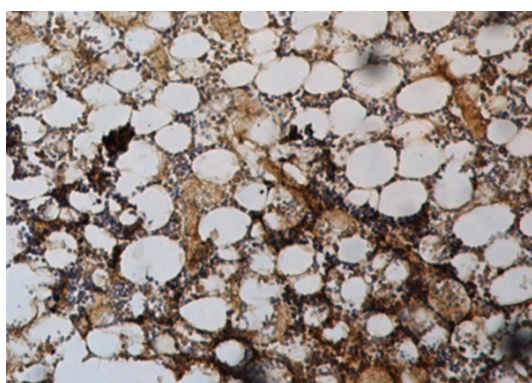

Figure 9. Type II collagen immunohistochemical staining of 2nd generation of cartilage cell after surgery for 4 months.

become one of the hot research topics in cell culture methods in recent years. There are some researchers who have confirmed that the passage chondrocytes can maintain some of the characteristics of chondrocytes in a long time under three-dimensional culture conditions [12]. There are also other experts who have found that chondrocytes can undergoredifferentiation and their phenotype can be restored even before they has not yet entered into the late differentiation [13]. At this point we need to know that with three-dimensional cultures, carriers with different materials of three-dimensional structure and cells with different types are co-cultured in vitro, cells can migrate, grow in three-dimensional spatial structure of the carrier, and eventually constitute the three-dimensional cell-carrier complex, including alginate medium culture support system, culture systems of cell plus scaffolds of cellulose absorbable polylactic acid or polymer,

etc. That is to say, it can provide the best micro-environment for cell growth.

As the three-dimensional framework of cartilage tissue engineering, cell scaffold can not only provide a frame for attachment of cartilage cells, which can form specific tissues or organs, but more importantly, they can be a part of the extracellular matrix components in cartilage tissue, and plays a role in mediating cell signaling and interaction. This would have important effects on the growth of cartilage. Researches of Endres, Gabler, et al. [14, 15] showed that PLA, PLGA and its derivatives can be used as the material of three dimensional carrier for cartilage tissue engineering, and furthermore, with excellent plasticity, they can also be applied to surgery and arthroscopic surgery. Bioceramic hard stents prepared by the raw materials of β-TCP with controlled microporous structures not only have good biocompatibility and high mechanical strength, but also controllable porous structure and degradation rate (both can be controlled according to the requirements of tissue growth [16]), as well as adjustable physical micro-structure (hole and the connector). When Ximin Guo et al. inoculated bone marrow mesenchymal stem cells onto prepared β-TCP, and then implanted them into a goat's articular cartilage defect, they found that prepared β-TCP possesses good biocompatibility and cartilage tissue growth guidance ability in vivo, and that the porous structure was suitable for cartilage tissue growth. That is to say, prepared β-TCP is the ideal scaffold material for cartilage tissue engineering [17]. Because it is implanted in the drilling of the cartilage, which can provide effective support that could allow

Table 3. Cartilage morphology score of 4 months after the operation

	Blank group	Primary cartilage	2 generation of rib cartilage
Average number	4.76±1.56	17.65±2.67	20.76±2.13

early load pressure, so it is beneficial to the repair of normal cartilage. As is known to all, the microstructure of the scaffold has a great influence on the cell adhesion and growth, and the bioceramic hard scaffold, which can be a unique feature of the different types of scaffolds due to its unique micro-structure (pore size and internal connection), its microstructures can be adjusted to a suitable condition the is beneficial to the cell's adsorption and proliferation [16]. Moreover, scaffold of high porosity is also beneficial to the migration and proliferation of bone marrow cells in the scaffold, and that is very important for the repair of bone and cartilage [18].

Autologous cartilage transplantation (ACI) technology has been used for a long time in clinical application, and it has demonstrated exciting early clinical results [19]. It is worth mentioning that ACI technology has experienced several reforms to overcome its shortcomings. By using molecular biology technology, researchers have used plasmid or adenovirus with a target gene transfected into bone marrow mesenchymal stem cells, and then induced them to differentiate into chondrocytes. They found that when cells are in the local release of growth factor, they undergoes differentiation at the same time [20]. Finally, it would differentiate into tissue engineering seed cells, which would in turn complete the tissue repair through the double effect of tissue engineering and molecular biology.

Despite its promising potential in clinical application, it is difficult to popularize the laboratory technology, even, there is a potential risk of cancer transfection. Hettrich CM et al. who worked in New York special department of orthopedics hospital in 2008, proposed that by using a cartilage matrix, allogeneic cartilage cells, and transplantation-friendly mechanical environment, could make ACI operations become possible [21], while there is also potential immune suppression by the use of allogeneic cartilage cells. In 2006, through compari-

son of the performances of the rib cartilage cells, articular cartilage cells, nasal septal chondrocytes, and auricular chondrocytes under monolayer culture and composite culture with poly L-lactic acid (PLLA), Isogai, N et al. found that it is most abundant in rib cartilage cells and nasal septum cells in terms of the rate of cell proliferation, expression of type II collagen and glycosaminoglycan expression and maintenance. This indicated that because of its large quantity and simple access, rib cartilage cells can be used as the seed cell source of articular cartilage injury and repair [22].

In this research, by studying the microstructure of -TCP porous ceramics, we found that porous bioceramic connections with an inner diameter of 120 μm and aperture of 500-630 μm are most suitable for the attachment and growth of chondrocytes. The reasons for the result may be that their suitable internal connections and pore size can regulate the relationship among attachment, growth of cartilage cell, secretion of extracellular matrix and growth factors, which can cause the rib cartilage cells to get the best nutritional conditions, and then secrete extracellular matrix and cytokines such as TGF-β and type II collagen that are necessary to its growth and phenotype maintaining, and finally to better maintain the growth and differentiation of chondrocytes.

In this animal research, we confirmed that the rib cartilage cells can be used as seed cells for the repair of articular cartilage defects, the second generation of which is more capable than the primary one. Moreover, cartilage cells combined with β-TCP bioceramic can repair the rabbit bone defects, and the histological score of the second generation of the rib cartilage cells in April was 20.76 + 2.13, while the staining of toluene, red and collagen II also showed that cartilage tissue was completely repaired. All of these proved that, a composite construction in which the surface layer of cartilage is repared by chondrocytes while the scaffold material promotes ossification, can be achieved by using the composite biological ceramic material.

The results of this study can be further developed and applied in clinical practice, so that patients with arthritis cartilage injury can be free from pains and sufferings. It has a positive

significance in the early repair of cartilage injury in osteoarthritis, which will prevent the progress of osteoarthritis and reduce medical expenses, thereby improving people's quality of life.

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