Allogenic chondrocyte/osteoblast-loaded β-tricalcium phosphate bioceramic scaffolds for articular cartilage defect treatment

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ABSTRACT

The medical community has expressed significant interest in the treatment of cartilage defect. Successful repair of articular cartilage defects remains a challenge in clinics. Due to the huge advantages of 3D micro/nanomaterials, 3D artificial micro/nano scaffolds have been widely developed and explored in the tissue repair of articular joints. In this study, chondrocyte/osteoblast-loaded β-tricalcium phosphate (β-TCP) bioceramic scaffold and chondrocyte-loaded β-TCP bioceramic scaffold were prepared by micromass stem cell culture and bioreactor-based cells-loaded scaffold culture for articular cartilage defect treatment. The results demonstrate chondrocyte and osteoblast can be successfully induced from allogeneic bone marrow stromal cells using micromass stem cell culture. Further, chondrocyte-loaded β-TCP scaffold and osteoblast-loaded β-TCP scaffold can be successfully prepared by bioreactor-based cells-loaded scaffold culture. Finally, the scaffolds are applied for Beagle articular cartilage defect treatment. The relative cartilage regeneration abilities on Beagle femoral trochlea were as follows: Chondrocyte/osteoblast-loaded β-TCP bioceramic scaffold group > chondrocyte-loaded β-TCP bioceramic scaffold group > β-TCP bioceramic scaffold. Therefore, micromass stem cell culture and bioreactor-based cells-loaded scaffold culture can be applied to prepare chondrocyte/osteoblast-loaded β-TCP bioceramic scaffold for articular cartilage defect treatment. It suggests allogenic chondrocyte/osteoblast-loaded β-TCP bioceramic scaffold could be potentially used in the treatment of patients with cartilage defects.

Introduction

Articular cartilage is a non-vascular tissue, which is mainly composed of chondrocyte and cartilage extracellular matrix. Injured articular cartilage is a common and increasing disease in the field of orthopedics, articular wear, trauma, etc [1]. It can lead to disability and reduced quality of life. Although articular cartilage injuries are common, injured cartilage has a limited ability to heal [2]. Articular cartilage injuries with a diameter of more than 4 mm cannot spontaneously heal. Reconstruction of articular cartilage defects with a large diameter still remains one of the most difficult problems for joint surgeons [3,4]. Currently, traditional conservative treatment is mainly applied for mild articular cartilage injuries and artificial joint replacement is mainly applied for severe articular cartilage injuries in clinics. These treatments have their disadvantages such as restricted activity, limited life of the server, and potential infection. Many types of research demonstrated chondrocyte transplantation is a promising method for articular cartilage defect treatment [5–7]. Therefore, it is necessary to explore the clinical use of chondrocyte-loaded tissue engineering artificial bone with good ability of chondrogenesis.

3D artificial micro/nano scaffolds have already developed for bone regeneration [8,9], load-bearing bone defect repair [10,11] and large osteochondral defects in the articular joints [12]. Autologous chondrocytes-loaded micro/nano β-tricalcium phosphate (β-TCP) bioceramic scaffold has been explored in the repair of osteochondral defects and showed good regeneration behaviors [13–15]. However, limited autologous chondrocyte availability, the need for additional surgery, the formation of donor site defects, donor site pain, possible blood loss and infection, and impracticability of utilizing autologous bone in the clinics have restricted their
potential application. Moreover, the application of allogenic chondrocyte is also not ideal in clinics because of many disadvantages for the donor person such as limited allogenic chondrocyte availability, the need for additional surgery, the formation of surgical defects, donor person site pain, possible blood loss and infection, and impracticability of utilizing allogenic chondrocyte in the clinics. Therefore, it is necessary to develop efficient allogenic stem cell sources and explore the preparation of chondrocyte-loaded 3D bioceramic scaffolds for articular cartilage defect treatment.

Bone marrow stromal cells (BMSCs) are important cell sources for bone tissue engineering [8,16]. BMSCs can be extracted from allogenic bone marrow by a minimally invasive surgery, which has many advantages for the donor person such as short treatment time, less blood loss, shorter recovery time, and shorter hospitalization compared with traditional surgical treatment [17,18]. They can be differentiated into a number of cell lineages such as chondrocyte and osteoblasts [19]. Human BMSCs-loaded 3D porous polymer scaffolds have been developed for cartilage tissue engineering [20,21]. A bioreactor has been developed for perfusing cells in 3D tissue engineered scaffolds and the results demonstrated that it was an efficient way to perfuse cells in 3D porous scaffolds [22-24]. 2D monolayer chondrocyte culture has a tendency to undergo dedifferentiation and have a higher probability to differentiate and form hypertrophic chondrocytes [25,26]. High density 3D micromass chondrocyte induction has been shown to secrete higher amounts of collagenous matrix and showed better cell differentiation behaviors [27,28]. Therefore, it would be a good idea to explore the combination of 3D micromass culture with bioceramic scaffolds. In this work, in order to obtain good treatment behavior of chondrocyte and osteoblast-loaded β-TCP bioceramic scaffolds for articular cartilage defect treatment, we try to explore the application of 3D micromass stem cell culture for chondrocyte and osteoblast induction prior to bioreactor-based cells-loaded scaffold culture.

**Materials and methods**

**Chemicals**

Percoll diluted solution was prepared by mixing 9 volume of Percoll solution (Pharmacia, Uppsala, Sweden) and one volume of 10X PBS. Complete cell culture medium consists of 90% low-glucose Dulbecco’s modified Eagle’s medium (L-DMEM, HyClone Inc, USA), 10% fetal bovine serum (FBS; Gibco, Carlsbad, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. Osteoblast induction solution was prepared by adding dexamethasone (100 μM), β-glycerol phosphate sodium (10 mM), and vitamin C (0.05 mM) into Hepes-buffered Dulbecco’s modified Eagle’s medium (H-DMEM, HyClone Inc, USA) with 0.05% FBS. Chondrocyte induction solution was prepared by adding bone morphogenetic protein-2 (BMP-2, 200 ng/mL), insulin-like growth factor 1 (IGF-1, 10 ng/mL), transferrin (6.25 μg/mL), dexamethasone (0.1 μM), and vitamin C (50 μg/mL) into H-DMEM with 10% FBS.

**Extraction, isolation, and culture of BMSCs**

In order to prepare BMSCs, three healthy Beagles with an age of ~4-10 months and a weight of 7.2–10.1 kg were used. The Beagles were anaesthetized. The wool on the Beagle iliac bone was shaved, and the areas to be used for the surgeries were disinfected with tincture of iodine. The iliac bone was punctured by using a skin incision trocar with a diameter of 1.5 mm. Then 6–7 mL bone marrow was extracted and stored in a 50 mL centrifuge tube with 0.4 mL Heparin (3000 U/mL) for each Beagle. The extracted bone marrow was mixed with D-Hanks solution. The supernatant and adipose tissues were removed. The solution was re-dispersed and was transferred into 1.073 g/mL Percoll diluted solution (3:2, v/v). The mixture solution was gently vortexed and then was centrifuged at 2800 rpm for 20 min. BMSCs were taken out and transferred into D-hanks solution (1:1, v/v). The mixture solution was gently vortexed and then was centrifuged at 1000 rpm for 8 min. The BMSCs were seeded in complete cell culture medium and cultured in a humidity-saturated incubator with 5% CO₂ at 37 °C. The culture medium was half-replaced on day 4 and fully replaced every 2 days after 7 days. The cell growth was examined by inverted optical microscopy. When cultured cells reached more than 90% confluence at day 12–14, adherent cells were detached with 0.05% trypsin-EDTA (Gibco, USA) and replated (passaged) at a density of 1 × 10⁶ per 175 cm² flask until processing for infusion [29].

**Micromass stem cell culture**

The third-passage cells were detached with 0.05% trypsin-EDTA and dispersed in complete cell culture medium at a density of 1 × 10⁶ per mL. 6 drops (100 μL per drop) of cell suspension was placed onto 60 mm petri dish with a space of 8 mm and was cultured in a humidity-saturated incubator with 5% CO₂ at 37 °C. The culture medium was half-replaced on day 4 and fully replaced every 2 days after 7 days. The cell growth was examined by inverted optical microscopy. Chondrocyte and osteoblast suspensions were prepared as follows: After 14 days of induction, the media in the chondrocyte and osteoblast groups were removed and washed by D-hanks solution three times. Then, the cells were detached with 0.05% trypsin-EDTA and dispersed in D-hanks solution in centrifugal tubes. The cell suspensions in centrifugal tubes were centrifuged at 1000 rpm for 8 min. The supernatants were removed and 2 mL H-DMEM with 10% FBS was added into the centrifugal tubes. The tubes were gently vortexed to prepare chondrocyte and osteoblast suspensions. The cell density was adjusted to 4 × 10⁶ per mL by adding an appropriate volume of H-DMEM with 10% FBS. After 14 days micromass culture, the chondrocyte induction group and control group were examined by glycosaminoglycan content analysis and cell viability assay. Glycosaminoglycan content analysis was performed using a
modification of the dimethylmethylene blue method [30]. The cell viability was examined by MTT assay [31].

Bioreactor-based construction of chondrocyte-loaded or osteoblast-loaded β-TCP bioceramic scaffolds

β-TCP bioceramic rods (Shanghai Bio-Ilu Biomaterials Co., Ltd., China) with a diameter of 0.45 cm and a height of 0.45 cm were put into the wells of 12-well cell culture plates and were disinfected by ultraviolet irradiation for 1 h and immersed into 75% ethanol overnight. Then, the bioceramic rods were washed by PBS six times and then immersed into H-DMEM overnight. Six bioceramic rods were randomly divided into two groups: (a) chondrocyte-loaded β-TCP bioceramic scaffold group; and (b) osteoblast/β-TCP bioceramic scaffold group. For each group, 2 mL cell suspension was added onto β-TCP bioceramic rod, which was turned upside down to make sure that cells attached on every surface of the rod. The rods were incubated in a humidity-saturated incubator with 5% CO2 at 37°C for 4 h. After that, the rods were put in a custom-designed bioreactor with a flow speed of 3 mL/min for H-DMEM with 10% FBS. The bioreactor was put in a humidity-saturated incubator with 5% CO2 at 37°C. The culture media were fully replaced every 6 days.

The scaffolds after 21 days of incubation were examined by JSM-T300 (JOEL, Japan) SEM. Briefly, the cells in the scaffolds were fixed by 2.5% glutaraldehyde (pH 7.2–7.4) for 30 min. The scaffolds were washed thrice by immersing in fresh PBS for 15 min. Then, the scaffolds were fixed by 1% osmium tetroxide solution (pH 7.2–7.4) for 60 min and were washed thrice by immersing in fresh PBS for 15 min. The fixed scaffolds were dehydrated by immersion in graded acetone solutions (30, 50, 70, 80, 85, 90, 100 and 100%) for 7 min in each solution. The scaffolds were immersed into dimethyl sulfoxide and then transferred into liquid nitrogen. After that, the scaffolds were dried under vacuum and were coated with an ultrathin gold layer. Finally, the scaffolds were observed by SEM at an accelerated volage of 10 kV. Three specimens were observed for each sample.

Implantation of bioceramic scaffolds into beagle femoral trochlea

In this work, healthy Beagles with an age of 10–12 months and a weight of 8.1–10.2 kg were employed as animal models. A total of 9 beagles were obtained from the Animal Center in Shandong Provincial Hospital Affiliated to Shandong University. The use of the Beagles and the experimental procedures in this study were approved by Shandong Provincial Hospital Affiliated to Shandong University’s Animal Care and Use Committee.

The Beagles were anesthetized and the wool on the interior of the knee joint was shaved. Then, the areas to be used for the surgeries were disinfected with tincture of iodine. The skin and subcutaneous fascia were incised. The knee joint was incised through the interior of kneecap. The kneecap tendon and quadriceps femoris tendon were stretched out. The kneecap was stretched to form dislocation and the femoral trochlea was exposed. Three cylinder-like articular cartilage defects with a diameter of about 0.45 cm and a depth of about 0.45 cm were constructed in the femoral trochlea. Chondrocyte/osteoblast-loaded β-TCP bioceramic scaffold, chondrocyte-loaded β-TCP bioceramic scaffold, β-TCP bioceramic scaffold were randomly implanted into these three defects of each femoral trochlea. Then, the kneecap was restored, knee capsule and muscular fascia tissue were sutured, and the skin incision was sutured. Gentian violet was spread on the skin incision. The Beagles were cultured at 15–20°C and received intramuscular injection of penicillin 800,000 IU once a day in the first three days to prevent infection. The Beagles (3 of each group) were sacrificed at 12 weeks. The treatments in femoral trochlea were evaluated by both naked eyes/digital cameras and histology.

Histological observation

The Beagles were sacrificed at 12 weeks postimplantation for histological observation. The femoral trochlea were harvested from the Beagle legs (the soft tissues were removed from the cartilages). The bioceramic scaffolds and the surrounding host cartilage tissues were excised and fixed in a 10% formalin solution at 4°C for 24 h. The specimens were decalcified in Formical-4 (forminal and formic acid; Decal Corporation, Tallman, NY, USA) for 24 h. After washing with water, fixed specimens were dehydrated by immersion in graded ethanol solutions (70, 75, 80, 85, 90, 95, and 100%) for 24 h in each solution. After dehydration, specimens were embedded in the medium of poly(methyl methacrylate) (Merck, Germany) and sectioned with a microtome (Leica, SP1600, Leica SP1600) equipped with sturdy tungsten carbide knives. The sections were polished and were imaged with a digital camera.

Results and discussion

Induction of chondrocyte and osteoblast from beagle BMSCs from marrow based on micromass stem cell culture

Healthy Beagles was applied to extract bone marrow. Then, BMSCs were isolated and were cultured. The primary cells (Figure 1(A)) and passage cells (Figure 1(B)) showed good cell shapes and good cell growth ability. The third-passage cells were detached from cell culture bottle wall for micromass stem cell culture. After 14 days micromass culture, the cells were examined by immunochemical staining observation (Table 1), MTT assay (Table 1), glycosaminoglycan content analysis (Table 1). Type II collagen is mainly produced by chondrocytes. Chondrocyte amounts were counted (Table 1) and showed significant differences between chondrocyte group and control group. Glycosaminoglycan is mainly secreted by chondrocytes. Glycosaminoglycan content analysis (Table 1) by a modification of the dimethylmethylene blue method showed significant differences between chondrocyte group and control group. Further, the MTT assay was applied to analyze cell viability. The results (Table 1) showed significant differences
between chondrocyte group and control group, which suggested the chondrocytes had good cell activity. Therefore, these results confirmed the successful induction of chondrocytes from BMSCs and the induced chondrocytes had good viability.

Preparation method of chondrocyte-loaded and osteoblast-loaded β-TCP bioceramic scaffolds using bioreactor-based cells-loaded scaffold culture method

A custom-design bioreactor system (Figure 2) was applied for the preparation of cells-loaded scaffolds [23]. It consists of a bioreactor vessel, peristaltic pump, air filter, three-way valve, media bottle, and two tubes to connect bioreactor vessel and media bottle. β-TCP bioceramic rods with a diameter of 0.45 cm and a height of 0.45 cm were applied as cell scaffolds. After 14 days of micromass culture of chondrocyte and osteoblast induction, the cells were detached and were explored for the preparation of cells-loaded β-TCP scaffolds. The chondrocyte-loaded β-TCP scaffold or osteoblast-loaded β-TCP scaffold were put in the bioreactor vessel. The media was forced to flow between the media bottle and bioreactor vessel by a peristaltic pump with a speed of 3.0 mL/min. When the system is assembled, it was put in a humidity-saturated incubator with 5% CO₂ at 37°C for the cells-loaded scaffold preparation.

After 21 days bioreactor-based culture, the cells-loaded β-TCP scaffolds were taken out and examined by scanning electron microscopy (SEM), as shown in Figure 3. β-TCP scaffolds without cell culture showed they had many pores with a diameter of 200–600 μm (Figure 3(C)) and the surface at micro-scale (Figure 3(D)) showed β-TCP scaffolds consisted of micro-particles with a diameter of 1–10 μm. This is consistent with the previous report about β-TCP scaffolds [32]. Chondrocytes adhered, spread, and proliferation well (Figure 3(A)) on the surface of β-TCP scaffolds. Osteoblast adhered, spread, and proliferation well (Figure 3(B)) on the surface of β-TCP scaffolds. Therefore, bioreactor-based cells-loaded scaffold culture method could be applied to successfully fabricate chondrocyte-loaded and osteoblast-loaded β-TCP scaffolds.

In vivo behaviors of allogenic chondrocyte/osteoblast-loaded β-TCP bioceramic scaffolds in beagle articular cartilage defect model

Bioreactor-based cells-loaded scaffold culture method was applied to prepare chondrocyte/osteoblast-loaded β-TCP scaffolds.
bioceramic scaffold and chondrocyte-loaded β-TCP bioceramic scaffold. After 21 days bioreactor-based culture, these scaffolds were applied to treat Beagle articular cartilage defect model.

Healthy Beagles were applied to prepare articular cartilage defect model. Three cylinder-like articular cartilage defects with a diameter of about 0.45 cm and a depth of about 0.45 cm were constructed in the femoral trochlea (Figure 4(A)). Then, chondrocyte/osteoblast-loaded β-TCP bioceramic scaffold, chondrocyte-loaded β-TCP bioceramic scaffold, β-TCP bioceramic scaffold were randomly implanted into these three defects (Figure 4(B)). Then, the kneecap was restored, knee capsule and muscular fascia tissue were sutured, and the skin incision was sutured. After 12 weeks of animal culture, the Beagles were sacrificed and the treatments in femoral trochlea were evaluated by both naked eyes/digital cameras and histology.

General observation of articular cartilage regeneration (Figure 4(C)) showed there was no evidence of swelling and effusion of the knee joints. There was also no evidence of infection on the knee joints. Moreover, no synovial hyperplasia and cartilage destruction were found in operative joints. However, chondrocyte/osteoblast-loaded β-TCP bioceramic scaffold group and chondrocyte-loaded β-TCP bioceramic scaffold group showed better articular cartilage regeneration ability than β-TCP bioceramic scaffold group.

Histological observation of these three groups was performed by naked eyes/digital cameras. As shown in Figure 5(A), chondrocyte/osteoblast-loaded β-TCP bioceramic scaffold group showed the new cartilage formed very dense structure in the defect area and connected very well to the surrounding host cartilage tissues (The borderline was not very clear). Compared with the surrounding cartilage and subchondral bone, the regeneration percentages of newborn cartilage and subchondral bone were 79 ± 10% and 91 ± 3%, respectively. As shown in Figure 5(B), chondrocyte-loaded β-TCP bioceramic scaffold group showed the defect depth was shortened and the new cartilage formed dense structure in the defect area. The borderline between defect (scaffold) and the surrounding host cartilage was clear. Compared with the surrounding cartilage and subchondral bone, the regeneration percentages of newborn cartilage and subchondral bone were 79 ± 10% and 91 ± 3%, respectively.

Figure 3. SEM images of cells on bioceramic scaffolds. (A) Chondrocytes on β-TCP bioceramic scaffolds. (B) Osteoblasts on β-TCP bioceramic scaffolds. (C) Section image of β-TCP bioceramic scaffolds. (D) Surface image of β-TCP bioceramic scaffolds.
cartilage and subchondral bone were 74 ± 5% and 70 ± 5%, respectively. As shown in Figure 5(C), β-TCP bioceramic scaffold group showed the defect depth was shortened and the new cartilage formed dense structure in the defect area. Compared with the surrounding cartilage and subchondral bone, the regeneration percentages of newborn cartilage and subchondral bone were 0% and 58 ± 4%, respectively. Moreover, the relative cartilage regeneration abilities on Beagle femoral trochleae were as follows: Chondrocyte/osteoblast-loaded β-TCP bioceramic scaffold group > Chondrocyte-loaded β-TCP bioceramic scaffold group > β-TCP bioceramic scaffold. It suggested the co-presence of both chondrocyte and osteoblast might be more suitable for cartilage regeneration.

In this work, the articular cartilage defect treatment was explored by allogenic chondrocyte/osteoblast-loaded β-TCP bioceramic scaffolds. Firstly, chondrocyte and osteoblast were successfully induced from dog BMSCs from allogenic marrow based on micromass stem cell culture. Then, chondrocyte-loaded and osteoblast-loaded β-TCP bioceramic scaffolds were successfully prepared by bioreactor-based cells-loaded scaffold culture method. Finally, Beagle articular cartilage defect model was constructed and the in vivo cartilage defect regeneration ability of allogenic cells-loaded β-TCP bioceramic scaffold was explored and they showed good regeneration ability. The relative cartilage regeneration abilities on Beagle femoral trochleae were as follows: Chondrocyte/osteoblast-loaded β-TCP bioceramic scaffold group > Chondrocyte-loaded β-TCP bioceramic scaffold group > β-TCP bioceramic scaffold. Moreover, compared with previous results using 2D monolayer chondrocyte culture [25,26], this work showed no obvious formation of hypertrophic chondrocytes. Therefore, chondrocyte/osteoblast-loaded β-TCP bioceramic scaffolds using 3D micromass culture are better choice for articular cartilage defect treatment. Chondrocyte/osteoblast-loaded β-TCP bioceramic scaffolds had several advantages for the treatment of cartilage defects: (i) Good cartilage regeneration ability; (ii) wide allogenic BMSCs availability; (iii) no need additional surgery for patients; (iv) no donor site defect formation in patients; (v) less possible blood loss and infection. This work suggested micromass stem cell culture and bioreactor-based cells-loaded scaffold culture can be applied to prepare chondrocyte/osteoblast-loaded β-TCP bioceramic scaffold. The allogenic chondrocyte/osteoblast-loaded β-TCP bioceramic scaffold could be potentially used to treat cartilage defects and could benefit patients with cartilage repair need.
It should be noted further work is needed to analyze the underlying mechanisms prior to clinical application.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This work was supported by the National Natural Science Foundation of China [30672116] and the National High Technology Research and Development Program of China [863 Program 2013AA032203].

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