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Interpenetrating polymer network scaffold of sodium hyaluronate and sodium alginate combined with berberine for osteochondral defect regeneration

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ABSTRACT

Degradation of the articular cartilage and structural remodeling of the subchondral bone are regarded as the two major pathological characteristics of osteoarthritis. This study aimed to investigate the effect of an interpenetrating polymer network (IPN) of a sodium hyaluronate and sodium alginate (HA/SA) scaffold combined with berberine (BER) on osteochondral repair. We first developed an IPN scaffold of HA/SA and evaluated its characteristics. Then, we analyzed the effect of the HA/SA scaffold combined with BER on the healing of osteochondral defects *in vivo*. Finally, we explored the mechanism of this system in osteochondral repair. The results showed that the system could simultaneously regenerate not only the cartilage but also the subchondral bone. Our results also revealed that the subchondral bone was partially repaired by activating the Wnt signaling pathway and the cartilage was protected from degeneration by the upregulation of autophagy. This study demonstrated that the combination of the IPN scaffold of HA/SA and BER is a promising strategy for the osteochondral defect regeneration.

1. Introduction

Osteoarthritis (OA), characterized by degradation of articular cartilage and degeneration of subchondral bone, is a destructive joint disease that can cause chronic pain and morphological changes [1]. Currently, no disease-modifying drugs are available, and the treatment is aiming at the amelioration of symptoms, or surgical replacement of joints is required. Degeneration of the cartilage and reconstruction of the subchondral bone are two major pathological characteristics of OA [2]. However, because the bone and cartilage tissues differ greatly in biological properties, fabricating a scaffold able to regenerate both tissues is of great importance.

Since the damage to the subchondral bone plays a pivotal role in OA, repairment of subchondral defects is of great importance. It has been reported that various types of scaffolds can repair osteochondral defects, including stratified and non-stratified scaffolds [3]. However, most scaffolds can only reconstruct either chondral or osseous tissues, and thus it is difficult for such scaffolds to simulate the natural structure of cartilage and subchondral bone [4]. There is currently no smart scaffold available that can simultaneously regenerate the subchondral bone and preserve the cartilage. Thus, it is important to develop a novel scaffold for the reconstruction of both osseous and chondral tissues.

Hyaluronan (hyaluronic acid, HA), a natural unbranched polysaccharide, is a major ingredient of the cartilage extracellular matrix, and it plays a multifaceted role in cell proliferation, morphogenesis, inflammation, and wound repair [5]. HA and its derivatives have been the focus of recent research in tissue engineering in wound healing and arthritis because of their biological functions and biocompatibility [6]. It has been reported that HA can promote the reconstruction of the cartilage and that injection of a cross-linkable HA hydrogel enhances the chondrogenesis [7]. Sodium alginate (SA), a polysaccharide copolymer, is also one of the most widely used materials for tissue engineering. SA has been investigated for cell encapsulation because of its biocompatibility and the ability to easily form a gel [8]. Previous study demonstrated that photocrosslinked alginate hydrogels were able to repair bone defects by delivering osteogenic materials [9]. Therefore, HA/SA might be a promising scaffold for the repairment of subchondral defects.

It has been proven that Wnt/β-catenin signaling pathway play a pivotal role in bone biology. Bone mass could be significantly increase by the upregulation of Wnt signaling pathway through a knockdown of Wnt antagonists or the overexpression of β-catenin [10]. In the process

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of activation of Wnt/β-catenin signaling, expression of the glycogen synthase kinase-3β/adenomatous polyposis coli/axin complex is downregulated, which leads to the accumulation of unphosphorylated cytoplasmic β-catenin. The unphosphorylated β-catenin is then transferred to the nucleus where it interacts with T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) to modulate the expression of downstream target genes [11].

Autophagy is crucial for the maintenance and remodeling of cellular homeostasis during normal cellular and tissue development [12]. Studies have revealed that autophagy plays an important role of a protective mechanism during cartilage degeneration. In the pathological articular cartilage in mice and humans, reduced expression of autophagy regulators can be observed [13]. By activating autophagy, the pathological progress of OA can be ameliorated [14]. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is constitutively expressed in normal human articular cartilage and is regarded as an effective marker of autophagy, owing to its expression both inside and outside of the isolation membrane during the autophagosome formation [15].

Berberine (BER) is an isoquinoline alkaloid present in many plants of the genera *Berberis* and *Coptis*. BER has been proven to exert significant immunosuppressive and anti-inflammatory effects [16]. Recently, it has been reported that BER can activate the Wnt/β-catenin signaling pathway in a rat model of OA [17]. The signaling molecules of Wnt pathway have been reported to play a vital role in osteogenesis, and thus we hypothesized that BER could upregulate the canonical Wnt signaling pathway to enhance the formation of subchondral bone. It has also been reported that BER enhanced the autophagy levels in glioblastoma, neurons, and a hypertrophic heart [18–20]. Our previous study has shown that degenerative OA changes were significantly ameliorated by the activation of autophagy [21], and we hypothesized that BER could protect the cartilage by increasing the autophagy level. Thus, based on the previous studies, BER has the potential to fulfill the biologically requirements for dual-regeneration of either chondral or osseous tissues in osteochondral defects.

To assess the therapeutic potential of IPN scaffolds of HA/SA combined with BER for osteochondral repair, we first developed an IPN scaffold of HA/SA and evaluated its characteristics. Then, we analyzed the effect of this scaffold, combined with BER, on the regeneration of osteochondral defects *in vivo*. We hypothesized that our IPN scaffold of HA/SA, along with BER, could promote the osteochondral repair through the dual-lineage effect of BER.

2. Materials and methods

2.1. Materials

Sodium hyaluronic acid (HA) (1040 kDa) and sodium alginate (SA) (medium viscosity, Mw = 250,000 g/mol, guluronic residue/mannuronic residue ratio = 30:70) issued from *Macrocystis pyrifera* were purchased from Shandong Freda Biochem Co., Ltd. (Jinan, China) and Sigma–Aldrich (St-Louis, USA), respectively. The crosslinker, poly (ethylene glycol) diglycidyl ether (PEGDG, n = 20, MW = 8886 Da), was purchased from Polysciences (Warrington, PA). Trypsin/EDTA, type II collagenase (290 units/mg) and isopropanol were purchased from Sigma–Aldrich (St-Louis, USA). BER (purity ≥98%) and XAV939 (inhibitor of Wnt/β-catenin pathway) were purchased from Sigma-Aldrich (St. Louis, USA). All other chemicals were of analytical grade purchased from Sigma-Aldrich (St-Louis, USA) unless specifically mentioned.

2.2. Preparation of the HA/SA IPN scaffold

Sodium hyaluronic acid/sodium alginate (HA/SA) IPN scaffold was prepared by mixing each polymer of HA/SA weight ratios 4:1 (w/w) in 0.1 N NaOH solution containing 1 ml of PEGDG. The reaction mixture was incubated at 60 °C for 6 h. The resulted hydrogels were swelled with 0.01 M calcium chloride solution for 18 h and freeze-dried in order to get the sponge form.

2.3. Characterization of the HA/SA IPN scaffold

To characterize the internal microstructures of the materials, the swollen hydrogel samples were frozen at −80 °C and lyophilized. The dried samples were mounted on aluminum stubs, sputter-coated with gold, and observed under a scanning electron microscope (SEM, Hitachi S3000N) at an accelerating voltage of 15 kV.

2.4. Isolation and culture of cells

Rabbit bone marrow-derived mesenchymal stem cells (BMSCs) were isolated from the bone marrow of four-month-old New Zealand White rabbits (n = 5) by gradient isolation of mononuclear cells, and they were cultured in Dulbecco's modified Eagle medium with high glucose (DMEM-HG) (Gibco) plus 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B at 37 °C in a humidified atmosphere that contained 5% CO_2. Cells were plated in T-75 flasks and were allowed to adhere for 24 h. Non-adherent cells were removed by three washes with PBS, and the adherent cells were cultured in complete DMEM-HG. Cells were detached using 0.25% trypsin-EDTA upon 80–90% confluence and were subcultured as passage one (P1). Mouse articular cartilage was obtained from the femoral condyles and tibial plateaus of C57BL/6 mice on postnatal day 5–6. Chondrocytes were maintained as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37 °C. Cells were utilized in the experiments through the 3rd passage.

2.5. Cell viability assay

The scaffold was immersed in 0.12 ml DMEM-high glucose containing 10% FBS and 1% penicillin/streptomycin for > 24 h. BMSCs were seeded on a 96-well plate on the scaffold at 10^3 cells/well. After 3 days, the cytotoxicity of the scaffold was assessed using Cell Counting KIT-8 (CCK-8, Dojindo, Kumamoto, Japan) at 450 nm. Cell number was correlated with optical density (OD).

Live and dead assay was used to observe the cell status in scaffolds by fluorescence microscope (Nikon Eclispse Ti-S). Calcein-AM (2 μM, Dojindo) and propidium iodide (10 μg/ml) were used to dye live cells and dead cells respectively.

2.6. Cell proliferation assay

Cell proliferation was measured using CCK-8. The BMSCs-seeded scaffold, at desired time points (1, 3, 5, and 7 days), was incubated in CCK-8 solution in a 5% CO_2 (v/v) incubator at 37 °C for 3 h. The intense orange formazan derivative formed by cell metabolism was soluble in the culture medium. The absorbance was measured at 450 nm. Cell number was correlated with OD.

2.7. In vitro BER release profile

To characterize the standard curve of BER, a suitable amount of BER was weighed to prepare different concentrations of BER solutions (5, 10, 20, 40, 80 mg/L). The optical density (OD) values of the BER solution samples were quantified using the spectrophotometer at 266 nm. BER was mixed with HA/SA IPN scaffold before testing. The *in vitro* BER release profile from the scaffold was determined at pH 7.4 with 0.01 M PBS solution. The amount of BER and scaffold was 0.4 mg and 40 μl. The mixture was placed in a 50 ml Eppendorf tube. Then, 20 ml 0.01 M PBS was added into the tube. The sample was incubated at 37 °C with gentle agitation. At the desired times (0, 1, 3, 6, 12, 18, 24, 36, 48, 60, and 72 h), the OD values of 100 μl aliquots were quantified using a