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Mesenchymal stem cells can survive on the extracellular matrix-derived decellularized bovine articular cartilage scaffold

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ABSTRACT

Objective (s): The scarcity of articular cartilage defect to repair due to absence of blood vessels and tissue engineering is one of the promising approaches for cartilage regeneration. The objective of this study was to prepare an extracellular matrix derived decellularized bovine articular cartilage scaffold and investigate its interactions with seeded rat bone marrow mesenchymal stem cells (BM-MSCs).
Materials and Methods: Bovine articular cartilage that was cut into pieces with 2 mm thickness, were decellularized by combination of physical and chemical methods including snap freeze-thaw and treatment with sodium dodecyl sulfate (SDS). The scaffolds were then seeded with 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate (DiI) labeled BM-MSCs and cultured for up to two weeks.
Results: Histological studies of decellularized bovine articular cartilage showed that using 5 cycles of snap freeze-thaw in liquid nitrogen and treatment with 2.5% SDS for 4 hr led to the best decellularization, while preserving the articular cartilage structure. Adherence and penetration of seeded BM-MSCs on to the scaffold were displayed by histological and florescence examinations and also confirmed by electron microscopy.
Conclusion: ECM-derived decellularized articular cartilage scaffold provides a suitable environment to support adhesion and maintenance of cultured BM-MSCs and could be applied to investigate cellular behaviors in this system and may also be useful for studies of cartilage tissue engineering.

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Introduction

Tissue engineering is a novel approach that applies principles to biology and engineering for the development of biological replacement that strives to maintain or improve tissue function in patients (1). The first and major component of tissue engineering is to provide a three-dimensional structure for the growth of cells, while maintaining their differentiated function is the scaffold (2). Various types of scaffolds including natural and synthetic have been used in tissue engineering both experimentally and in clinical applications. Many research lines have revealed that naturally derived- scaffolds derived from decellularized tissues and organs are functionally superior to synthetic polymers (3, 4). They usually retain their biocompatibility and may evade the immune response, because their xenogenic or allogenic cellular antigens are removed. Furthermore, due to preservation of most of the structural and functional proteins in the scaffold, they provide signals, which facilitate cell attachment (5). (extracellular matrix)ECM, which is composed of a rich meshwork of proteins and proteoglycans, is a main communication layer between the environment and cells (6). ECM could be harvested from animal tissues and be used directly as scaffolds for tissue engineering applications. ECMs derived from a variety of tissues have been used as biological scaffolds, including heart valves (7), blood vessels (8), skin (9), skeletal muscle (10), tendons (11), small intestinal submucosa (SIS) (12, 13), urinary bladder (14, 15) and liver (16). In this study, decellularized bovine articular cartilage was prepared and used as a scaffold.

To do so, multiple treatments with sodium dodecyl sulfate (SDS) were applied in order to find the best structural properties, in which decellularization was achieved while collagen and glycosaminogelycans (GAGs) contents were preserved. Self-repair of cartilage lesions is difficult due to the low regenerative capacity and blood supply of cartilage, and tissue engineering as an interdisciplinary field is on the lookout for cartilage repair (2). The main goal of the decellularization protocol is the best elimination of

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all cellular and nuclear materials, while minimizing any adverse effects on the composition, biological architecture and function of the remaining ECM (17). However, cartilage is a compact tissue, which is difficult to remove its cell components completely (2). SDS is an ionic detergent, which permeabilizes the cytoplasmic and nuclear membranes and has been used to decellularize other musculoskeletal tissues including meniscus, temporomandibular joint, tendon and ligament (11, 18-20).

The source of cells is another important component of tissue engineering strategies. Bone marrow-derived mesenchymal stem cells (BM-MSCs) considered as a promising candidates for cartilage tissue engineering due to their ability of proliferation, multiple differentiation and ease of harvest (5). These multipotent cells can be expanded and differentiated to obtain connective tissue cells like bone, cartilage, muscle, tendon and fat (21-24). The objectives of this study was to produce a decellularized bovine articular cartilage scaffold, which retains the main ECM molecules by a combination of physical and chemical methods and also to investigate the interaction of this scaffold with seeded BM-MSCs.

Materials and Methods

Preparation of ECM-derived cartilage scaffold

Animal experiments were performed according to the Iranian Council for the Use and Care of Animals Guidelines and were approved by the Animal Research Ethical Committee of Ferdowsi University of Mashhad. In this experimental study, articular cartilages of femur bovine bones were harvested from the sacrificed animals immediately after slaughter and were cut into coin-like pieces with 5 mm diameter and 2 mm thickness. To decellularize the articular cartilage tissue, a combination of physical and chemical methods was applied. For physical decellularization, specimens were maintained at -4 ˚C for a week and then after thawing and washing with normal saline, snap freeze-thaw in liquid nitrogen were performed for 5 cycles. In order to perform chemical decellularization, specimens were treated with 2.5% SDS (Merck, Darmstadt, Germany) for 1, 4 and 8 hr at 37 ˚C to achieve the best elimination of cells, while preserving the extracellular matrix components. Then, the specimens were rinsed in phosphate-buffered saline (PBS) for 30 min. In the next step, in order to remove SDS and minimize potential contaminations, the decellularized scaffolds were placed in a sterile Buchner funnel and washed with 75% ethanol, sterile distilled water and PBS, respectively.

Evaluation of the decellularized bovine articular cartilage scaffold by histological methods

Paraformaldehyde 4% (Merck, Darmstadt, Germany) was applied to prepare specimens for histological and fluorescence studies. After fixation, specimens were dehydrated through a graded series of ethanol, embedded in paraffin (Lab-O-Wax, Milan, Italy), cross-sectioned at a thickness of 5 µm with a microtome (Leits, Vienna, Austria), deparaffinized, rehydrated and stained with hematoxylin-eosin (H&E) (Merck, Darmstadt, Germany) to determine the construct cellularity. Safranin-O (Merck, Darmstadt, Germany) (25) and toluidine blue (Merck, Darmstadt, Germany) stainings were performed to demonstrate the GAGs content in decellularized ECM. Picrosirius-red (Merck, Darmstadt, Germany) staining was also employed to examine the effects of decellularization on the collagen-rich ECM. Using polarizing microscope (Olympus, IX70, Japan) for observing the sections stained with picrosirius-red is a specific method to identify the collagenous structures (26). DNA contents were stained with 4, 6 diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Taufkirchen, Germany), which is a fluorescent dye that binds to nucleic acids with high affinity for DNA.

Bone marrow-derived mesenchymal stem cells (BM-MSCs) isolation and expansion

To derive rat BM-MSCs, the bone marrow of a one-month old male Wistar rat was collected and the cells were transferred into a cell culture flask filled with 5 ml Dulbecco's modified Eagle's medium (DMEM, Gibco, Paisley, Scotland) supplemented with 15% fetal bovine serum, (FBS, Gibco, Scotland) and 100 µl penicillin/streptomycin (Biosera, Sussex, UK). Cells were then incubated at 37 ˚C and 5% CO_2 in air. After the removal of blood and stromal cells, BM-MSCs were subcultured and purified by trypsinization (0.25% trypsin /EDTA (Ethylenediaminetetraacetic acid) solution, Biosera, Sussex, UK) for four times (27, 28).

Cell labeling, seeding and culture method

To confirm the detection of BM-MSCs on the scaffolds, the cells were labeled before seeding. 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate (DiI, Invitrogen, Karlsruhe, Germany) that stains the cytoplasm of cells was used to label BM-MSCs. To prepare the stock solution of DiI, 50 µg of DiI was dissolved in 50 µl dimethyl-sulfoxide (DMSO, Merck, Darmstadt, Germany), and 1 µl of the stock solution was added to 1 ml PBS. Then the medium was removed and 1 ml of the prepared dye was added to a 25 cm^2 flask. The cells were incubated at 37 ˚C for 5 min and then transferred to -4 ˚C for 10 to 15 min. Fresh medium was then added to each flask. The cells were incubated overnight and then applied for seeding.

DiI stained BM-MSCs at passages 4 were trypsinized, counted and the scaffolds were seeded with 2×10^5 cells/scaffold in 2 ml complete medium, and specimens were placed in 24-well plates.

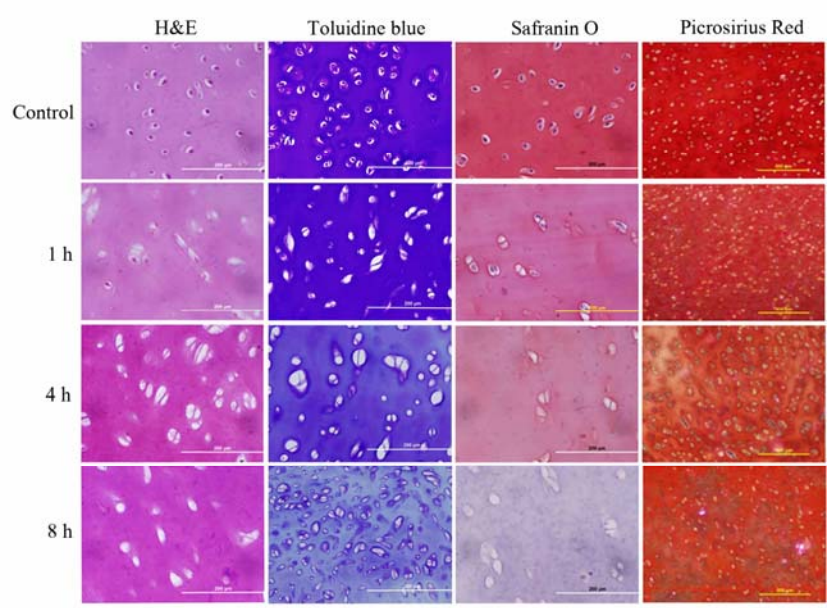

Figure 1. Control and decellularized articular cartilage specimens. Construct cellularity, glycosaminoglycan (GAG) and collagen content have been displayed in various decellularization groups compared with native cartilage. H&E staining demonstrated that treatment with 2.5% SDS for 1 hr has led to incomplete elimination of cellular components while treatment for 4 and 8 hr eliminated cell nuclei and components completely. Toluidine blue and Safranin-O staining demonstrated the decrease of GAG content and Picrosirius Red staining displayed decrease of collagen content in specimens treated for 8 hr. Decellularized specimens treated for 4 hr have shown the best elimination of cellularity while preserving of GAG and collagen contents

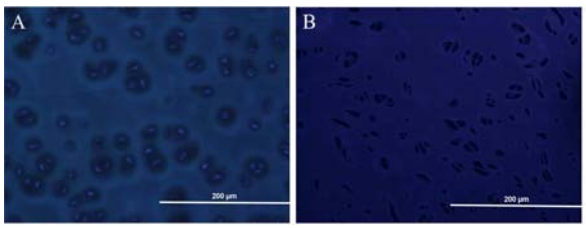

Figure 2. Staining of bovine articular cartilage with 4, 6 diamidino-2-phenylindole (DAPI) before and after decellularization. Native bovine articular cartilage combined with bright nuclei representative of the presence of intact cells in native tissue (A). Elimination of cell nuclei in obtained decellularized scaffold (B) compared with native articular cartilages is displayed, which proved the efficiency of decellularization procedure

Seeding was performed in a dropwise fashion onto the scaffold. Seeded scaffolds were incubated in a humidified atmosphere at 37°C with 5% CO_2 in air. The medium was changed every 2 to 3 days. Some unseeded scaffolds were cultured as controls. Histological analyses were performed after 3, 7 and 14 days of culture.

SEM analysis

In order to prepare samples for electron microscopy, the specimens were fixed with 2.5% glutaraldehyde (TAAB Laboratories Equipment Ltd. Reading, UK) for 24 hr followed by three 15 min washes in 0.1 M sodium cacodylate buffer (pH 7.4, TAAB Laboratories Equipment Ltd. Reading, UK). Then, the specimens were treated with 1% Osmium tetroxide (TAAB Laboratories, UK) for 1 hr and washed in 0.1 M sodium cacodylate buffer. At the next step, the sections were subjected to sequential dehydration with 30%, 50%, 70%, 90%, and absolute ethanol solutions, followed by three washes with absolute ethanol for 15 min. Then the specimens were fixed on metal stubs and coated with gold-palladium by sputtering (Sputter coater, SC7620, Sussex, UK) and examined under a scanning electron microscope (SEM; LEO 1450VP, Oberkochen, Germany).

Results

Characterization of the decellularized articular cartilage by histological studies

All specimens were harvested from bovine articular cartilage, cut into coin-like pieces and subjected to physical decellularization and then chemical treatments with 2.5% SDS for 1, 4 and 8 hr. The obtained decellularized scaffolds were evaluated histologically.

H&E staining of native and decellularized articular cartilage groups revealed that treatment with SDS for 1 hr resulted in incomplete decellularization of articular cartilages, while in other groups treated for longer period, better elimination of cells was obtained. Using safranin-O and toluidine-blue stainings that indicate the distribution of sulfated proteoglycans and GAGs in the ECM, showed an extensive reduction in stained GAGs in specimens treated for 8 hr, which reveals qualitative loss of GAGs after decellularization. Specimens stained by Picrosirius Red also demonstrated the preservation

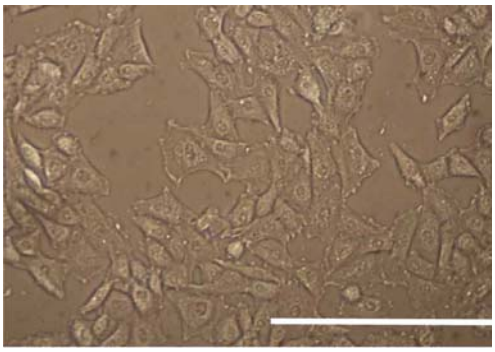

Figure 3. Morphology of the monolayer cultured rat bone marrow derived mesenchymal stem cells at passage 4 (scale *bar*=200 µm)

of collagen fibers in decellularized samples treated with SDS for 1 and 4 hr, while collagen content of scaffolds treated for 8 hr displayed extensive decrease after decellularization (Figure 1). Therefore, the specimens treated with 2.5% SDS for 4 hr appeared to be the best group to achieve a decellularized articular cartilage scaffold with the best elimination of cells and most preservation of ECM contents.

Furthermore, DAPI staining also confirmed the elimination of nuclear DNA in obtained decellularized scaffolds as shown in Figure 2. In agreement with results of light and fluorescence microscopy, all cells had been removed, and the decellularized cartilage tissue could be observed in the SEM images (Figure 5).

Investigation of interactions between BM-MSCs and decellularized articular cartilage scaffold

DiI labeled BM-MSCs at passage 4 were used for seeding onto the decellularized articular cartilage scaffolds (Figure 3).

After 24 hr of seeding, some scaffolds were stained with methylene blue and as it is shown in Figure 4, blue spots on scaffolds verified the viability of seeded cells. SEM images of seeded scaffolds also demonstrated the adherence of BM-MSCs to the scaffolds after culture (Figure 5).

The adhesion and maintenance of seeded cells were also investigated after 3, 7 and 14 days of culture. As shown in Figure 6, H&E staining of cultured scaffolds indicated the attachment and penetration of seeded MSCs into the lacuna of decellularized articular cartilage.

Fluorescence microscopy of specimens can be observed in Figure 7. Seeded articular cartilage scaffolds with DiI labeled BM-MSCs were examined after DAPI staining at 3, 7 and 14 days of culture. Labeled BM-MSCs were detected as brightly spots located inside the lacuna of decellularized articular cartilage scaffolds.

Discussion

In recent decades, the tissue engineering has been progressing rapidly. One of the most important

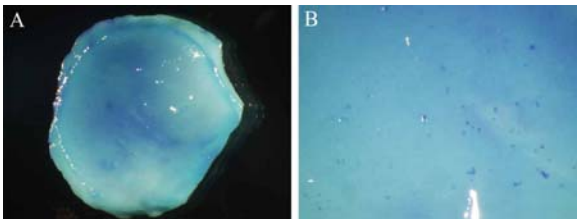

Figure 4. Methylene blue staining of seeded scaffolds after 24 hr of culture (A, ×15 & B, ×70 magnification)

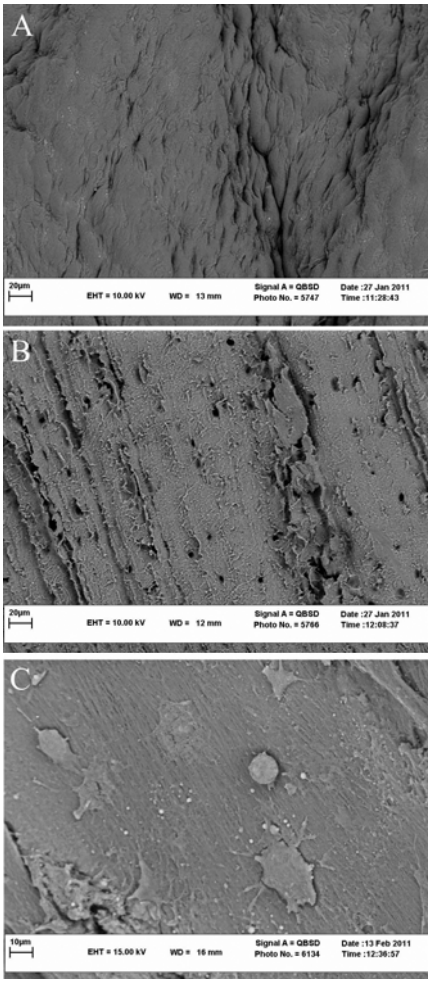

Figure 5. A) Scanning electron micrographs of the native articular cartilage tissue B) Decellularized articular cartilage scaffold and C) Penetration and adherence of bone marrow mesenchymal stem cells on the scaffold after 48 hr of culture is displayed.

factors in cartilage tissue engineering is the preparation of a scaffold (29). To achieve an optimal scaffold in cartilage tissue engineering, development and application of scaffolds derived from natural and synthetic materials have been performed. So far, various decellularization methods have been suggested to develop extracellular matrix derived scaffolds (30, 31). To prevent the immune response,

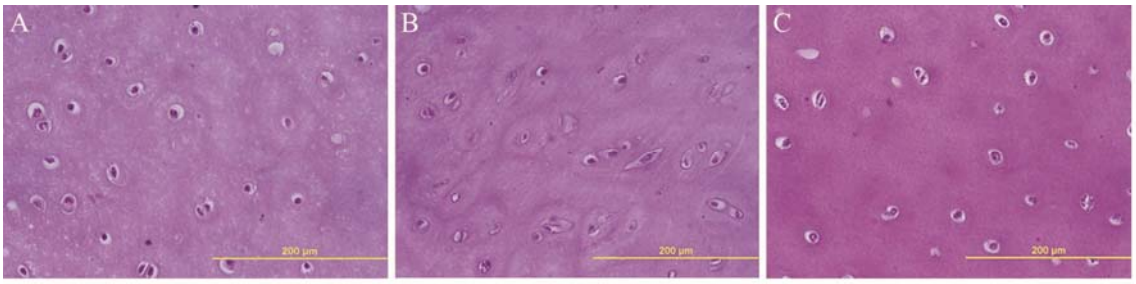

Figure 6. Hematoxylin-eosin staining of seeded scaffolds after 3, 7 and 14 days of culture, which display adherence and maintenance of bone marrow mesenchymal stem cells into lacuna of decellularized scaffold during culture

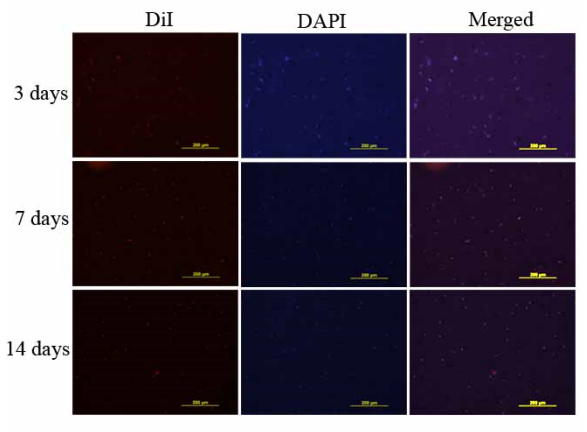

Figure 7. Penetration and adherence of 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate (DiI) labeled bone marrow mesenchymal stem cells into lacuna of decellularized scaffold were detected after 4, 6 diamidino-2-phenylindole (DAPI) staining at 3, 7 and 14 days after culture by fluorescent microscope

it is essential that antigenic epitopes are removed from intra and extracellular matrix (32, 33). Several investigations have reported that snap freeze-thaw cycles followed by treatment with various detergents would lead to removal of cellular components from tissues (19). In this study, in order to decellularize the articular cartilage, a combination of both physical and chemical methods was used. Snap freeze-thaws cycling in liquid nitrogen were used for 5 cycles as the physical method. Studies have shown that although the freeze-thaw cycling disrupts the cells and intracellular proteins, some cellular components such as cell nuclei would not be removed by this process and treatment with other reagents is necessary to remove cell components completely (34). Several detergent based methods are developed to decellularize various tissues and organs (35). The combination of detergent treatments with freeze-thaw shows a synergistic effect on removal of cellular components and leads to a greater success in decellularization (5, 19, 36, 37). In this study, treatment with 2.5% SDS for different time periods was employed to achieve the best elimination of cellular components with preservation of extracellular contents. Although using 2.5% SDS for 1 hr did not damage the extracellular matrix, many cellular components were remained in the tissue. On the other hand, treatment with 2.5% SDS for 4 hr led to complete omission of cellular components with perseveration of extracellular matrix contents, while 8 hr treatment led to the loss of histochemical characteristics of the ECM.

Studies have demonstrated that removal of DNA is relatively difficult compared to other intracellular components due to its sticky properties and strong tendency to adhere to ECM proteins (17). DNA remaining after decellularization process might be a reason for inflammatory reactions following implantations (38, 39). Thus, it is important to use more specific methods for detection of DNA. DAPI staining demonstrated complete elimination of DNAs from the obtained decellularized cartilage tissue. Furthermore, microstructures of our obtained scaffolds were analyzed and compared with native articular cartilage using electron microscopy and confirmed elimination of cells from the decellularized tissues. Therefore, it can be concluded that a combination of freeze-thaw cycles and treatment with 2.5% SDS for 4 hr would lead to a maximal removal of cells along with relative preservation of GAGs and collagen contents of the cartilage ECM.

In order to assess the ability of obtained ECM derived scaffold to support the attachment and growth of the cells, rat BM-MSCs were seeded onto the scaffolds. Using these cells has several advantages including the presence of non-invading methods for their harvest (especially from adipose tissue), and also they have great potential for differentiation into osteocytes, chondrocytes and adipocytes (40). These cells were initially labeled with a cytoplasmic fluorescent dye and DiI and then were used to seed the scaffolds. Methylene blue staining of scaffolds after 24 hr of culture confirmed the existence of living cells on the scaffolds, which can be observed as blue spots. SEM micrographs showed the adhesion of BM-MSCs onto the scaffold. Maintenance of seeded cells was also demonstrated during longer culture periods. It is interesting that most of the seeded cells were located within the lacuna that might indicate the ability of BM-MSCs to adhere and adapt to the scaffold. These

results are in agreement with Yang *et al* study who demonstrated the adhesion, proliferation and differentiation of seeded MSCs labeled with fluorescent dye PKH26, to chondrocytes on decellularized human joint scaffold (5).

Conclusion

An effective method for decellularization of each tissue and organ depends on several factors such as thickness, density and cellularity of the tissue. Any change in the ECM characteristics due to decellularization process may have effect on behavior of seeded cells. Therefore, more investigations are required to optimize the preparation process of decellularized scaffolds for tissue engineering applications.

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