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# Scaffolds derived from cancellous bovine bone support mesenchymal stem cells' maintenance and growth

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**Abstract** Since bone defects can lead to various disabilities, in recent years, many increasing attempts have been made in bone tissue engineering. In this regard, scaffolds have attracted a lot of attention as three dimensional substrates for cell attachment which improve successful tissue engineering. The aim of the present study was to provide an interconnected porous scaffold to facilitate cell infiltration. To do so, cancellous bone from bovine femur was dissected in fragments and decellularized by physicochemical methods, including snap freeze/thaw, rinsing in hot water and treatment with different solutions of sodium dodecyl sulfate (SDS). Histological analysis and 4′,6-diamidino-2-phenylindole staining revealed that the best results were obtained after treatment with 2.5%, 5%, and 8% SDS for 8, 3, or 1 h respectively, which significantly removed bone cells with intact trabeculae geometry. Further characterization of decellularized scaffolds by the compression tests also revealed no significant difference between elastic modulus values of the three different SDS treatments. Moreover, studying the ratio of bone trabeculae to bone surfaces (BT/BS) as assessed by Clemex vision software 3.5 showed that treatment with 2.5% SDS for 8 h resulted in a BT/BS score in the range of native bone and therefore this treatment was used for further experiments. Histological studies and scanning electron microscopy revealed rat mesenchymal stem cells integration, adhesion, and maintenance during the 2 and 7 d of culture in vitro. In conclusion, the present results support the effective role of SDS in cancellous bovine bone decellularization and also propensity of treated samples in providing a suitable three-dimentional environment to support the maintenance and growth of mesenchymal stem cells.

**Keywords** Cancellous bone · Decellularization · Scaffold · Mesenchymal stem cells

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## Introduction

Critical bone defects resulting from trauma, cancers, nonunion fractures and abnormal skeletal developments, are all severe health challenges that need to be repaired by bone grafts. Autografts, allografts and xenografts are three types of bone grafts with limited clinical applications. Autografts suffer from donor site morbidity, size limitations, continuous pain after operation, hypersensitivity, and infection, while allografts are followed by severe immune response, low quantity, and possible virus infection (Kneser et al. 2006; Meijer et al. 2007; Abousleiman et al. 2008). However, bone tissue engineering is a promising approach to surpass the inadequate clinical treatments for bone

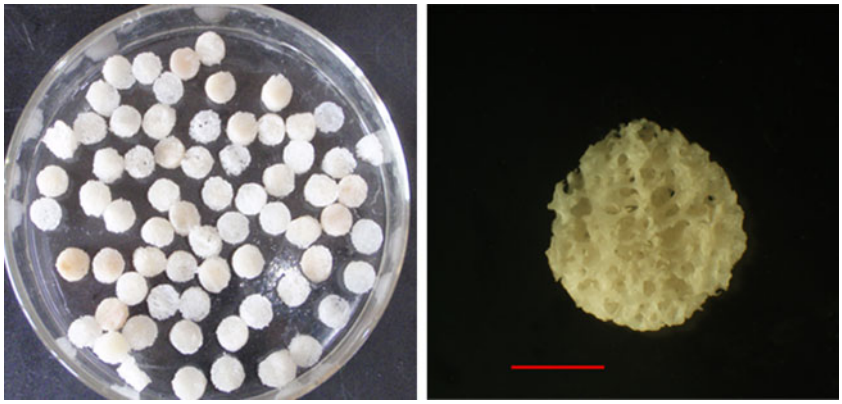

**Figure 1.** Macroscopic view of cancellous bone cylindrical pieces prepared from epiphyseal of bovine femur bone. (scale *bar*=2.4 mm).

injuries (Chao et al. 2007; Meijer et al. 2007). In this regard, scaffolds create a suitable environment for cellular behaviors such as proliferation, differentiation and migration, and can promote new tissue formation (Fröhlich et al. 2008; Tabata 2009). Among all natural and synthetic scaffolds, decellularized xenografts show several advantages including minimized immunogenic reactions, while preserving biological activities due to their unique architecture properties similar to those of the native tissues, which provide cues for recellularization (Elder et al. 2009; Wang et al. 2010; Vavken et al. 2012). Xenogeneic extracellular matrix (ECM) scaffolds have been created from decellularization of many tissues including porcine small intestine, tendon (Cartmell and Dunn 2000), heart valves (Liao et al. 2008), bladder (Rosario et al. 2008) and urinary bladder submucosa (Badylak et al. 1989; Gilbert et al. 2005). Derivation of ECM scaffolds from cancellous bones is also desirable due to their osteoconductive and osteoinductive characteristics, ease of application and highly porous structure, that all result in rapid revascularization in vivo and a suitable environment for cells in vitro (Fröhlich et al. 2008). However, decellularization of native tissues, which reduces the immunogenicity of xenografts, can alter the tissue composition. To overcome this drawback, several chemicals have been introduced as decellularizing agents including non-ionic detergents, ionic detergents, and various enzymes (Lumpkins et al. 2008; Vavken et al. 2012). Triton X-100 is widely used as a non-ionic detergent for decellularization but it has displayed various results. When it was used for decellularization of heart valves, the complete decellularization was not observed. It means the cellular materials remained in the myocardium and aortic wall (Grauss et al. 2005). Other studies showed that it is not an effective detergent for removing cells from blood vessels, tendon and ligament (Woods and Gratzer 2005). Moreover, Triton X-100 resulted in loss of glycosaminoglycans (GAGs) completely (Grauss et al. 2005). The tensile strength of collagen fibers were also severely altered after exposure to Triton X-100 (Gilbert et al. 2006). The best efficiency of using Triton X-100 can be achieved when it is used in combination with other decellularization protocols (Gilbert et al. 2006). In comparison with other agents, sodium dodecyl sulfate (SDS) has a long-standing record as an ionic detergent for decellularization of tissues (Vavken et al. 2009). It disrupts proteins by unfolding these molecules and completely removes nuclear and cellular remains. Although SDS led to decrease of GAGs concentration, it does not remove collagen fibers from tissues (Gilbert et al. 2006). Moreover, bovine pericardium was decellularized by SDS treatment which revealed a reduction in DNA content (Hülsmann et al. 2012). Another study showed that SDS was able to effectively remove cellular materials from adult porcine kidneys while it did not have any cytotoxic effects on human renal cells after repopulation. Thus, treatment with SDS maintained the structural properties and cellular compatibility of this renal scaffold (Sullivan et al. 2012). Some tissues such as male calves cartilage, ovine esophagus, and heart were also successfully decellularized by SDS treatment which preserved extracellular matrix (Ott et al. 2008; Ackbar et al. 2012). Since there is no report demonstrating the effects of SDS on cancellous bovine bone, we aimed to describe a decellularization method based on a novel physicochemical protocol. To assess the accuracy of decellularization protocol, histological staining and mechanical tests were used, and the biocompatibility of the porous scaffold was studied by the culture of rat mesenchymal stem cells (rMSCs) in vitro.

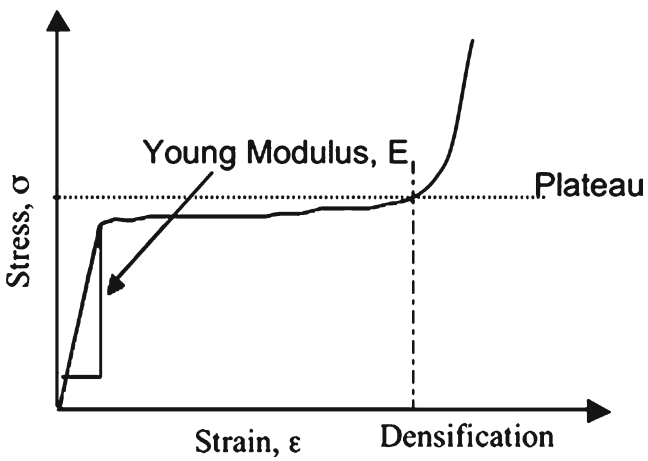


**Figure 2.** Schematic drawing of stress–strain compressive curve.

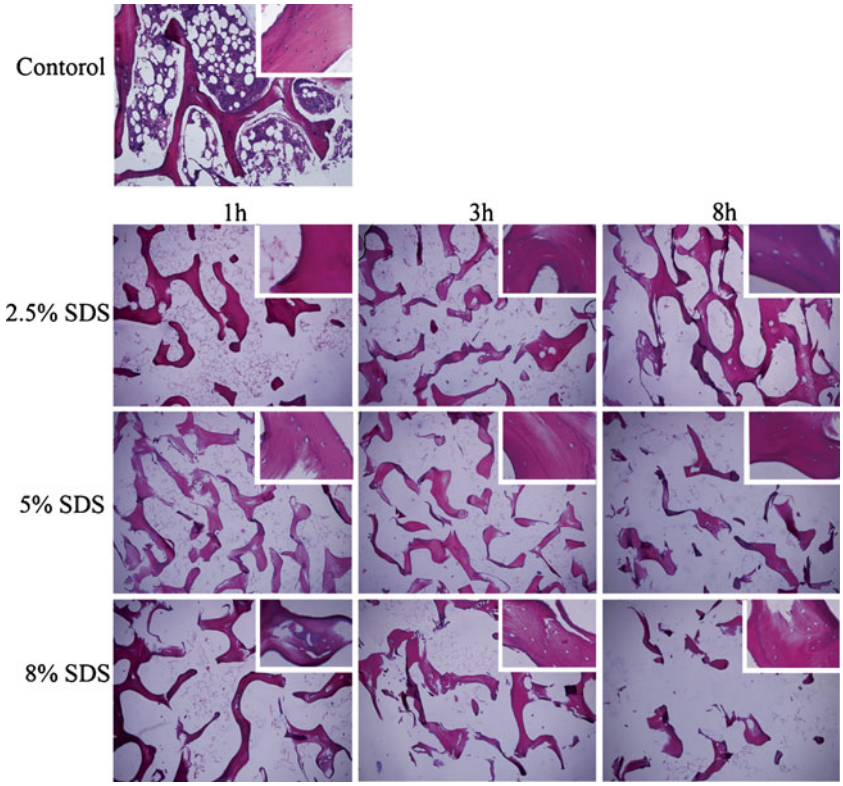


**Figure 3.** H&E staining of cancellous bones treated with different solutions of SDS. Treatment with 2.5% SDS or 5% SDS for 1 h and also 2.5% SDS for 3 h resulted in incomplete decellularization. Although complete removal of cell nuclei were obtained in treatments with 8% SDS for 3 or 8 h and 5% SDS for 8 h, significant changes in the architecture of the ECM were observed. Full decellularization and preservation of the ECM structure was observed after treatment with 2.5%, 5% and 8% SDS for 8, 3, or 1 h respectively (×100 magnification).

## Materials and Methods

*Cancellous bone preparation and decellularization process.* All experiments were performed according to the principle ethics for animal experimental investigations from Ferdowsi University of Mashhad. Cancellous bone was cut out from epiphyseal of bovine femur (10-12 month old, Holstein male calves), immediately after slaughter and dissected in cylindrical shapes with 5 mm diameter and 2 mm height (Fig. 1). The scaffolds were prepared from eight calves in total. Bone pieces were washed with sterile phosphate-buffered saline (PBS), rinsed in hot water five times (4 min each time) for up to 20 min to remove lipids, and stored at −4°C before decellularization. Frozen pieces were then thawed at room temperature (RT) and washed with sterile PBS, immersed in liquid nitrogen (−196°C) for 2 min and thawed in distilled water and then PBS at RT for 10 min. The freeze/thaw process, which leads to cell lysis, was repeated five times. In the chemical phase of decellularization, specimens were treated with different solutions of SDS (Merck, Germany) including 2.5%, 5%, and 8% (*w*/*v*) for 1, 3, and 8 h periods at 37°C, accompanied with gentle agitation to diffuse SDS into the tissues uniformly and remove the cell debris more effectively. Then, in order to reduce residual SDS from the scaffolds and sterilize them, two washing steps were used. In this respect, specimens were placed in a sterile Buchner funnel (Ilmabop, Germany), washed first with 75% ethanol to remove residual SDS from tissues and a second wash with PBS was employed for 30 min at room temperature to complete the decellularization process.

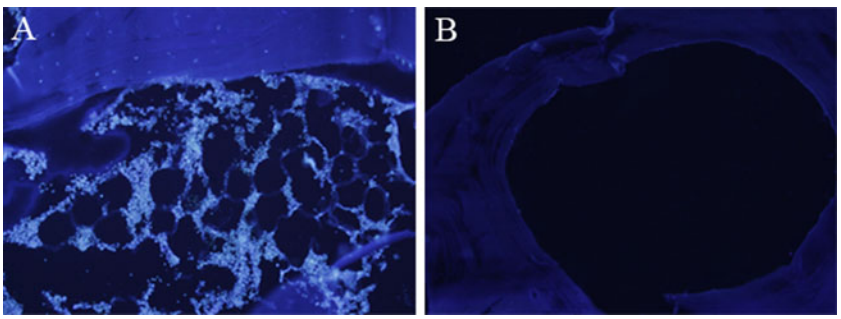


**Figure 4.** DAPI fluorescent staining of control group and a scaffold treated with 2.5% SDS for 8 h. (*A*) Normal bovine cancellous bone associated with bright nuclei indicates the presence of intact cells in bone tissue (×200 magnification). (*B*) Decellularized tissue with no sign of cells or cell fragments which proved the efficiency of decellularization procedure (×200 magnification).

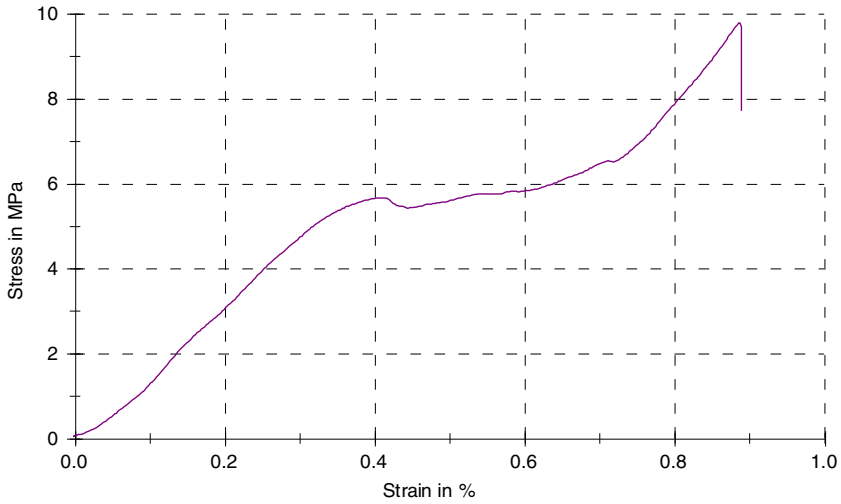


**Figure 5.** Representative stress–strain curve for control specimen tested at a strain rate of 0.03 mm/min. Example of Quasi static test without marrow.

*Mechanical testing.* Four cylindrical specimens of bovine cancellous bone prepared in 2-mm thickness were used for this test. Single-axis pressure test was performed and the specimens were compressed at a strain rate of 0.03 mm/min with a universal machine Zwick Z250 device (Zwick Company, Germany). Results are shown as stress versus strain for strain rate. The compressive modulus of each treatment was taken to be the slope of the stress–strain curve (shown as a schematic diagram in Fig. 2).

*Derivation of rat bone marrow mesenchymal stem cells.* To derive rMSCs, the bone marrow of a 1-mo 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, New York, NY) supplemented with 15% fetal bovine serum (FBS, Gibco, Netherlands) and 100 µl penicillin/streptomycin (Biosera). Cells were then incubated at 37°C and 5% $CO_2$ in air. After the removal of blood and stromal cells, rMSCs were subcultured and purified by trypsinization (0. 25% trypsin/EDTA solution, Biosera, UK) for four times.

**Table 1.** Compressive testes of control and decellularized cancellous bone scaffolds. All specimens are made by 5 mm diameter and 2 mm height (strain rate 0.03 mm/min)

| Specimen ($n$=4) | $E$ modulus (GPa) | | | |
|---|---|---|---|---|
| Control | 2.01 | 1.89 | 1.94 | 2.05 |
| 2.5% SDS—1 h | 1.75 | 1.84 | 1.90 | 1.79 |
| 2.5% SDS—3 h | 1.81 | 1.75 | 1.78 | 1.72 |
| 2.5% SDS—8 h | 1.21 | 1.16 | 1.02 | 1.07 |
| 5% SDS—1 h | 1.55 | 1.70 | 1.64 | 1.60 |
| 5% SDS—3 h | 1.0 | 1.14 | 1.22 | 1.05 |
| 5% SDS—8 h | 0.83 | 0.79 | 0.91 | 0.74 |
| 8% SDS—1 h | 0.99 | 1.08 | 1.04 | 0.96 |
| 8% SDS—3 h | 0.68 | 0.73 | 0.75 | 0.71 |
| 8% SDS—8 h | 0.67 | 0.59 | 0.65 | 0.64 |

*Cell seeding and culture method.* rMSCs in passage four were detached from the culture flask by 0.25% trypsin, centrifuged, and resuspended in appropriate volume of media to reach the density of $1\times10^6$ cells/ml. After sterilization, decellularized scaffolds were transferred in 24-well plates and seeded with 200 µl aliquots containing $2\times10^5$ cells and incubated at 37°C for 1 h to allow cell attachment. In the final step, seeded scaffolds were immersed in 2 ml complete medium, which was changed every 3 d. For each time point, unseeded scaffolds were used as controls, and all samples were subjected to histological staining and scanning electron microscopy at days 2 and 7 after cell seeding.

*Histological studies.* All samples were fixed in Bouin's solution and dehydrated through a graded series of ethanol, embedded in paraffin, cross-sectioned at a thickness of 7 µm with a microtome (Leits, Vienna, Austria), deparaffinized by xylene, rehydrated, and stained appropriately. To determine construct cellularity, hematoxylin and eosin (H&E), and DAPI (4,6-diamidino-2-phenylindole, Merck, Germany) stainings were used.

*Scanning electron microscopy.* To better observe decellularized and seeded scaffolds, they were fixed with 2.5% glutaraldehyde (TAAB Laboratories, UK) for 24 h, followed by several washing steps in 0.1 M sodium cacodylate buffer (pH7.4, TAAB Laboratories, UK). Then, samples were treated with 1% osmium tetroxide (TAAB Laboratories, UK) for 1 h, washed again in 0.1 M sodium cacodylate buffer, and dehydrated through a graded series of ethanol. Finally, scaffolds were fixed on metal stubs, coated with gold–palladium by sputtering (Sputter coater, SC7620, East Sussex, UK), and examined under scanning electron microscopy (SEM) (LEO 1450VP, Germany).

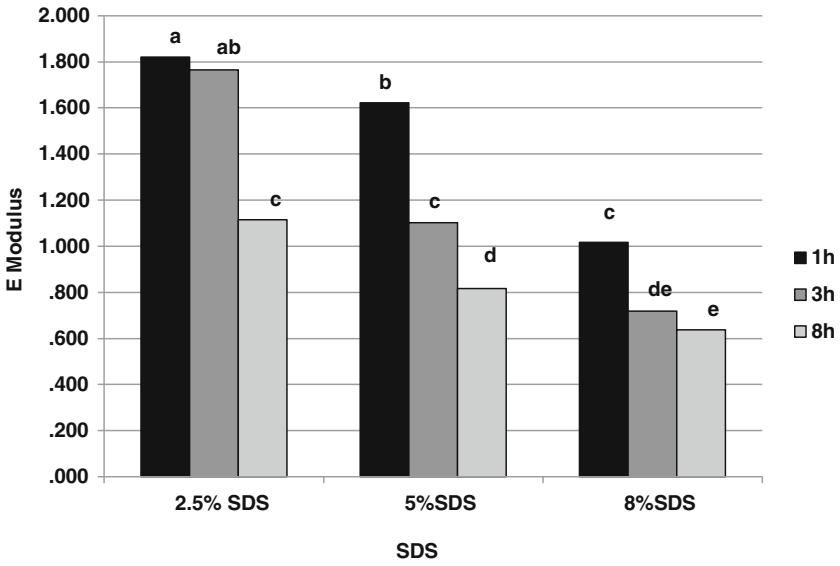


**Figure 6.** Young's modulus analysis during single-axis pressure test for treatments with various solutions of SDS at different times. Treatments with 2.5% SDS for 1 or 3 h and also 5% SDS for 1 h increased Young's modulus (*ab* with *a* and *b*). While treatments with 5% SDS for 8 h and 8% SDS for 3 or 8 h decreased Young's modulus (no significant difference between *de* with *d* and *e*). Slight decrease in Young's modulus was seen in treatments with 2.5%, 5%, and 8% SDS, for 8, 3, and 1 h, respectively. Different *letters* indicate a significant difference between groups ($p < 0.05$).

*Structural analysis.* In order to identify the structural properties of treated cancellous bone, and also better examine the effects of various solutions of SDS and time periods, all treated samples were imaged and analyzed by Clemex vision software 3.5 to measure the ratio of bone trabeculae to bone surface (BT/BS).

*Statistical analyses.* Statistical analyses were performed using SPSS (Version 19) and Minitab (Version 15) software. To assess significant differences between modulus value and various percentages of SDS and time points, Sidak analysis of variance (ANOVA) was used, while a $p$ value$<0.05$ was considered as significant.

## Results

*Preparation of decellularized scaffolds.* To obtain decellularized scaffolds, specimens were exposed to different solutions of SDS for 1, 3, and 8 h. Histological evaluations using H&E staining as shown in Fig. 3 clearly revealed the presence of various cell types including osteocytes and adipocytes after treating with 2.5% SDS for 1 h, 2.5% SDS for 3 h and also 5% SDS for 1 h. Moreover, treatments with 8% SDS for 3 h, similar to treatments with 5% and 8% SDS for 8 h, completely removed the nuclei, while resulting in significant changes in the architecture of the ECM. However, treatments with 2.5%, 5% and 8% SDS for 8, 3, or 1 h, respectively, resulted in complete removal of cells and maintenance of bony structures. The scaffolds prepared by treatment with 2.5% SDS for 8 h were also analyzed by DAPI staining in order to visualize double stranded DNA in them. As shown in Fig. 4, intact cells can be observed in controls, while, there was no sign of intact cells or cell fragments after decellularization.

**Table 2.** Analysis of decellularized and control specimens by Clemex vision

| Specimen | BT% | BS% |
|---|---|---|
| Control | 51 | 49 |
| 2.5% SDS—1 h | 21 | 79 |
| 2.5% SDS—3 h | 25 | 75 |
| 2.5% SDS—8 h | 36 | 64 |
| 5% SDS—1 h | 29 | 71 |
| 5% SDS—3 h | 20 | 80 |
| 5% SDS—8 h | 17 | 83 |
| 8% SDS—1 h | 25 | 75 |
| 8% SDS—3 h | 23 | 77 |
| 8% SDS—8 h | 12 | 88 |

*Mechanical properties of the scaffolds.* Bovine cancellous bone exhibited foam-type behavior (plateau border) under compression test. In this way, stress–strain curve for control specimen is shown in Fig. 5. This curve displayed a first linear elastic region and a plateau in the quasi-constant stress region. The stress–strain behavior in a uniaxial compression test was carried out to determine the apparent elastic moduli that were determined from the maximal slope within the "linear" region of the stress–strain curve. As a corollary, mean value, standard deviations and Young's modulus of various decellularization treatments are displayed in Tables 1 and 3.

*Modulus analysis.* The modulus values for different solutions of SDS and time points were measured during single-axis pressure. ANOVA test revealed significant differences between various solutions of SDS and time points with the modulus value ($E$). These results show that higher solutions of SDS in a long time, including 5% SDS for 8 h as well as

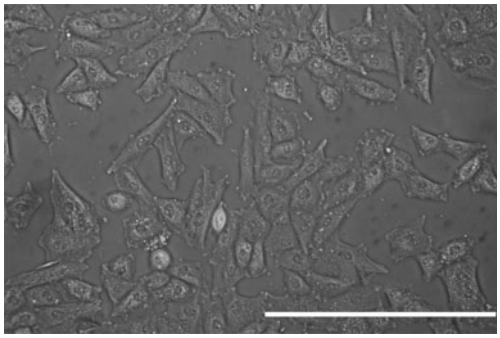

**Figure 7.** Morphology of rat bone marrow mesenchymal stem cells at passage four (scale *bar*=200 μm).

8% SDS for 3 and 8 h significantly reduced the mechanical properties of bone-derived scaffolds. As it is shown in Table 1, there was no significant difference in Young's modulus between treatments of 2.5% SDS for 1 and 3 h and also 5% SDS for 1 h with control specimens. On the other hand, treatments with 2.5%, 5%, or 8% SDS for 8, 3 or 1 h, respectively, slightly decreased Young's modulus (Fig. 6).

*The effects of structural properties on mechanical characteristics.* As it is presented in Table 2, analyzing specimens by Clemex vision revealed that among all treatments, only treatment with 2.5% SDS for 8 h resulted in a BT/BS (36/64) ratio closer to control samples (51/49). According to structural properties, treatment with 2.5% SDS for 8 h provided a proper substrate for cell seeding.

*rMSCs attachment and viability.* With respect to histological studies and biomechanical abilities, bone-derived scaffolds treated with 2.5% SDS for 8 h were chosen to be seeded with rat bone marrow-derived rMSCs at fourth passage (Fig. 7) (Edalatmanesh et al. 2010). rMSCs were seeded on the scaffolds for up to 7 d. H&E staining on sections of seeded scaffolds demonstrated that rMSCs were distributed between bone trabeculae at day 2 post-seeding (Fig. 8*A*), and increased numbers of rMSCs were detected 7 d after incubation (Fig. 8*B*). To confirm the results, the scaffolds were imaged by SEM before and after cell seeding. Figure 9 shows that the control scaffold is completely cell-free and presents a porous architecture of decellularized bone, while cells were adhered onto the surface of bone trabeculae 7 d after seeding.

## Discussion

Although bone has an excellent ability for repair by natural mechanisms, in certain clinical situations such as extensive injuries, congenital malformations or diseases, the natural restoration of bone may be too slow or inadequate. Therefore, in such cases, bone reconstruction with some kinds of grafting is recommended (Staubli et al. 2003; Fröhlich et al. 2008). Cancellous bone is exclusively used to fill the defects in order to enhance bone formation. Autologous cancellous bone has some advantages for clinical usage, including availability and safety. However, this approach has its own drawbacks, for instance, the harvest of autologous tissue can result in morbidity at the donor site, difficulty in the process of harvesting and post-operative continuous pain are not deniable (Fröhlich et al. 2008). In this regard, bone tissue engineering and the application of porous scaffolds have the potential to overcome the rising demands for suitable autograft and allograft tissues for reconstruction of very large bone defects (Meijer et al. 2007; Hutmacher 2001). Decellularization of the allogenic tissues upon scaffold transplantation is a crucial step, in order to minimize the immune response and reduce the risk of disease transmission. Since there are promising results of xenografts derived from several animal tissues (Greenwald et al. 2001; Kim et al. 2004; McFetridge et al. 2004; Ketchedjian et al. 2005), we aimed to decellularize cancellous bovine bone as a potential xenograft scaffold for studying bone tissue engineering. In comparison with other resources, bovine cancellous bone has been regarded as an appropriate model for tissue engineering due to its similarity in Haversian organization to human bone, higher metabolic activity than cortical bone, availability in large quantities from epiphyseal of bovine bone and also its osteoconductive and osteoinductive characteristics (Valentin et al. 2008). To achieve an applicable scaffold, whole cell bodies need to be removed, while leaving the structure and function of decellularized matrix unaffected. Thus far, a wide variety of decellularization methods have been used based on

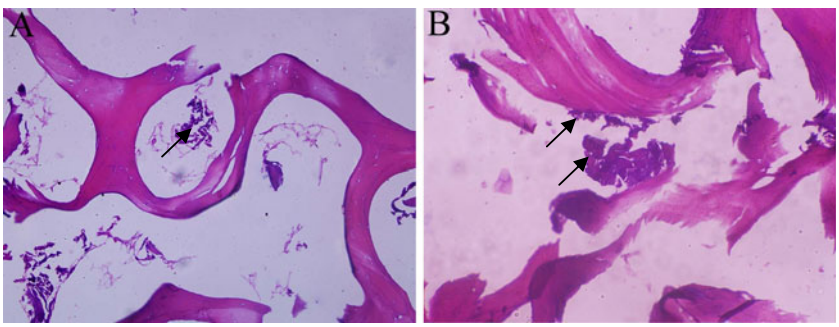


**Figure 8.** H&E staining of seeded scaffolds. (*A*) Presence of low densities of rMSCs (*arrows*) between bone trabeculae after 2 d and (*B*) the increase and attachment of cells as observed 7 d after culturing (×200 magnification in *A* and ×400 in *B*).

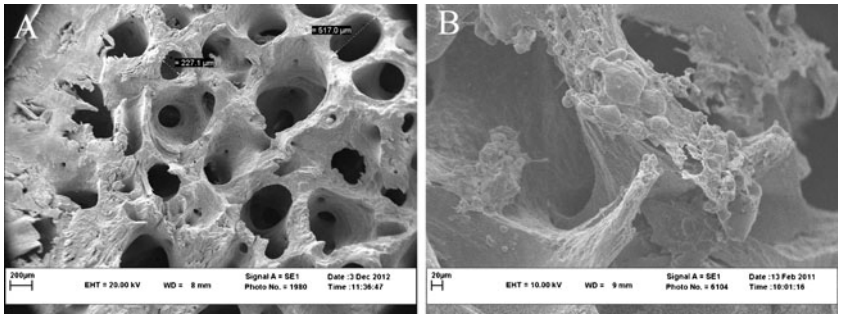


**Figure 9.** SEM images of (*A*) decellularized cancellous bone scaffold and (*B*) scaffold seeded with rMSCs. Cell attachments are obvious on bone trabeculae.

physical, enzymatic, and chemical treatments (Gilbert et al. 2006). Physical methods including snap freezing have been used widely for decellularization of tendinous and ligamentous tissues (Jackson et al. 1990). In this case, the membrane of cells is disrupted by rapidly freezing the tissues. Controlling the rate of temperature variation can prevent the ice formation and disruption of the ECM. It is an effective method for cell lysis but must be followed by other processes to remove the cellular materials from the tissues (Gilbert et al. 2006). A variety of chemicals have been utilized for decellularization, including non-ionic (Triton X-100) and ionic detergents (SDS). In some cases, SDS has shown excellent decellularizaton properties with maintenance of the ECM molecules and stiffness (Grauss et al. 2005; Rosario et al. 2008; Elder et al. 2009). For example, it was the most effective reagent for decellularization of articular cartilage, adult porcine kidney, bovine pericardium and ovine esophagus (Elder et al. 2009; Ackbar et al. 2012; Hülsmann et al. 2012; Sullivan et al. 2012). However, so far no study has demonstrated the decellularizing capacity of SDS in the bone. To determine the appropriate conditions for SDS treatment, various solutions and time periods were tested. To do so, bone specimens were first rinsed in hot water to eliminate the adipocytes, and then were exposed to repeating cycles of freeze/thaw in liquid nitrogen. Finally, all specimens were treated with various solutions of SDS in different periods of time with agitation to effectively diffuse the detergent through the cancellous bone. H&E staining was performed to assess the quality of decellularization. Additionally, single-axis pressure test by measuring the compressive modulus was employed. Our results revealed that treatments with 2.5%, 5% and 8% SDS, for 8, 3, and 1 h, respectively, resulted to complete decellularization with slightly decreased in Young's modulus, while in other conditions a number of cells were still present after histological examination or the structure of ECM was disrupted (Table 1, Figs. 3 and 6). Previous studies have shown that the increase in Young's modulus in shorter treatment periods is due to transient effects of SDS on collagen architecture. Under these circumstances, SDS disrupts non-covalent bonds in collagen fibers and the return of these deformed fibers to their native conformation, leads to increase in the Young's modulus at shorter incubation times (Otzen 2002). In addition, the presence of bone marrow has an apparent influence on mechanical behavior of cancellous bone (Chaarif and Drazetic 2007). To investigate the best structure geometry of decellularized bone, Clemex software was used to measure the bony trabeculae versus the bone surface BT/BS. Results revealed that treatments with 2.5% and 5% SDS for 1 h and also treatment with 2.5% SDS for 3 h, led to the BT/BS ratios as 21/79, 29/71, and 25/75, respectively. Although these treatments resulted in Young's modulus closer to that of native tissue (2.05 GPa), they revealed incomplete decellularization and their ratio of BT/BS was close to half of that resulted in native tissue (51/49), which indicates changes in the structure. Furthermore, treatments with 8% SDS for 3 and 8 h and 5% SDS for 8 h resulted in complete removal of cells as indicated by H&E staining and also significantly reduced Young's modulus and the ratio of BT/BS (Figs. 3 and 6, Tables 1 and 2). Despite complete decellularization by 5% and 8% SDS treatments for 3 and 1 h with slightly decreased in Young's modulus, they revealed changes in structural properties by measuring the BT/BS (Figs. 3 and 6, Tables 1 and 2). Since treatment with 2.5% SDS for 8 h resulted in complete elimination of cells (Fig. 3), a better BT/BS ratio and an appropriate Young's modulus, this SDS solution and time period were chosen for preparation of the scaffolds for further experiments.In order to remove the remaining SDS, which has a cytotoxic effect on cellular proliferation in vitro, the scaffolds

**Table 3.** *E* modulus analysis for different solutions of SDS (mean± standard error)

| SDS | Time | Mean | Std. error | 95% Confidence interval | |
|---|---|---|---|---|---|
| | | | | Lower bound | Upper bound |
| 2.50% | 1 h | 1.820 | 0.032 | 1.755 | 1.885 |
| | 3 h | 1.765 | 0.032 | 1.700 | 1.830 |
| | 8 h | 1.115 | 0.032 | 1.050 | 1.180 |
| 5% | 1 h | 1.623 | 0.032 | 1.557 | 1.688 |
| | 3 h | 1.103 | 0.032 | 1.037 | 1.168 |
| | 8 h | 0.818 | 0.032 | 0.752 | 0.883 |
| 8% | 1 h | 1.018 | 0.032 | 0.952 | 1.083 |
| | 3 h | 0.718 | 0.032 | 0.652 | 0.783 |
| | 8 h | 0.638 | 0.032 | 0.572 | 0.703 |

were washed three times with 75% ethanol and then PBS by Buchner funnel (Gratzer et al. 2006). To confirm the efficiency of decellularization, the histological properties of the scaffold were also verified by DAPI staining, which confirmed complete removal of the cells (Fig. 4*B*). Studies with SEM also indicated the microstructure of decellularized scaffold with a suitable interconnected structure which facilitates cell migration and the transportation of nutrients and waste during cell culture (Fig. 9*A*). The maintenance of cultured rMSCs in the scaffold was evaluated at days 2 and 7 after cultivation of the cells on the scaffold (Fig. 8). SEM images also proved successful attachment of cells on the bone trabeculae at 7 d after culture (Fig. 9*B*). These findings show that cell-free cancellous bone-derived scaffold prepared by physical and chemical decellularization methods can support cells attachment and maintenance. Therefore, this scaffold represents a promising construct for future bone tissue engineering as it has the capacity to successfully host rMSCs and also supports their vital functions such as attachment and proliferation. However, further studies are required to assess the possible immune response of the host tissue to this decellularized scaffold and also its potential to be used with some factors such as bone morphogenetic proteins in animal models. Overall, these results support the potential of an acellular porous cancellous bovine bone as a 3D microenvironment desired for 3D cell culture in vitro. It may be useful as a valuable xenograft model for repair of bony defects in human due to structural similarities to human bone, although further in vivo studies are required to confirm this (Table 3).

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