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# Dense Bone Derived 3D Bioscaffold: Preparation, Characterization, and Assessment of its Potential for Bone Marrow Mesenchymal Stem Cells (BM-MSCs) Growth and Differentiation

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## Abstract

**Background:** Since the bone defects can result in different disabilities, many efforts have been made to the bone tissue engineering. In this case, scaffolds play an important role as a key element of tissue engineering in providing three-dimensional structure for cell growth *in vitro*.

**Objectives:** The aim of present study was to provide the three-dimensional biological bioscaffold from the bovine femur dense bone and investigate the possibility of its potential for application in tissue engineering as biological 3D ECM bioscaffold via mesenchymal stem cells seeding and differentiation toward bone tissue.

**Methods:** For preparation of bioscaffolds, after cutting bovine femur bone into small pieces, demineralization and decellularization was done. Bioscaffolds biocompatibility was evaluated using MTT assay. The morphological and cell adhesion characteristics of Bone marrow mesenchymal stem cells (BM-MSCs) on the bioscaffolds were evaluated using Scanning Electron Microscopy (SEM) technique. Finally, the cells were treated with osteogenic differentiation medium and then evaluated for differentiation.

**Results:** Histological studies showed that the use of sodium dodecyl sulfate (2.5%) for 8 h eliminated the cells. Radiography and calcium oxalate test confirmed demineralization. MTT assay and SEM studies showed that the obtained bioscaffolds are biocompatible and could provide optimum three-dimensional environment for cell adhesion and movement. Moreover, the Alizarin red staining showed a higher differentiation rate for BM-MSCs

**Conclusion:** In the present study, bone derived 3D bioscaffoldshowed an important rolein the growth and differentiationof BMSCs, due to the natural characteristics, cell adhesion properties and potential to enhance differentiation of toward bone tissue. It may have potential for use as bioscaffold as supporting metrics for maintenance, growth in bone tissue engineering.

**Keywords:** Bioscaffold, Decellularization, Demineralization, Tissue engineering, Cell differentiation.

## Introduction

Severe bone defects resulting from trauma, cancers, nonunion fractures, and abnormal skeletal changes are considered major health challenges requiring repair by bone grafts.[1] The prevalent therapeutic techniques, i.e. allograft, xenograft, and autograft, have greatly improved the quality of these patients life. However, the mentioned methods also have a number of limitations such as the availability of donors, infection, weak fusion, and potential rejection.[2,3,4,5] Nevertheless, bone tissue engineering is a promising method to improve inadequate clinical treatments for bone injuries. Scaffolds, cells, and growth factors make up the three essential pillars of tissue engineering.[6]

As mammalian cells are dependent on adhesion and would not stay viable in case of lack of any proper substrate for cellular adherence, it is necessity to use appropriate scaffolds and substrates for cell culture.[7] Both biological and synthetic materials play an important role in tissue repair and engineering.[8] These compounds produce microenvironments that are similar to those of the extracellular matrix (ECM) of the tissue and guide the differentiation and formation of the target tissue.[9,10,11] The main challenge of bone tissue engineering is to specify the most suitable scaffold, which requires being biocompatible, biodegradable and compatibility for osteoconduction. In order to meet the mentioned requirements, several organic and inorganic biomaterials as well as their hybrids have been extensively examined.[12,13] Low mechanical properties, absence of biocompatibility, chronic inflammation, and immune responses are a number of the problems specified for the

existing scaffolds.[14] Biological scaffolds containing ECM have been proved to be applicable as a suitable substrate for cell growth and proposed as the most analogous scaffold to the main tissue. Allogeneic and xenogeneic ECM scaffolds are obtained from many tissues including heart valves,[15] veins,[16] nerves,[17] tendons and ligaments.[18] The most effective factors in the process of decellularization of a tissue and organ depends on several factors, including tissue cellularity (e.g. the liver versus the tendon), density (e.g. dermis versus adipose tissue), lipid content (e.g. the brain versus the bladder), and thickness (e.g. dermis versus pericardium). It should be noted that the selection of any method and agent for cell removal could alter the ECM compounds and, partly impair its ultrastructure. Minimizing these undesirable effects, in comparison with its complete avoidance, is the goal of decellularization.[19] Specialized techniques have been developed for decellularization, and the removal of cellular components is carried out through in corporating different physical, chemical, and enzymatic procedures.[20,21]

Non-ionic detergents are widely applied in decellularization because these compounds have a natural effect on tissue structure.[22] Triton X-100 is extensively applied as a non-ionic detergent for decellularization. Regarding the use of Triton X-100 for decellularization of the heart valves, complete removal of nuclear materials along with the thorough maintenance of the valve structure is observed.

However, cellular materials remain in the myocardium and aortic wall. According to the components of ECM, Triton X-100 results in complete loss of glycosaminoglycans (GAGs) and reduces the content of laminin and fibronectin in tissues.[23] Other studies have revealed that Triton X-100 is not effective in the complete removal of cellular materials from the veins, tendons, and ligaments [24,25,26] Sodium dodecyl sulfate (SDS), sodium deoxycholate, and Triton X-200 are the most commonly used ionic detergents.[21] Compared to other factors, SDS has been introduced as an ionic detergent suitable for decellularization of tissues.[27] SDS results in more perfect elimination of nuclear residues and cytoplasmic proteins (e.g., vimentin).[24] Although SDS decreases the concentration of GAGs, it cannot eliminate collagen fibers.[21] The decellularization of some tissues including the male calf cartilage, esophagus, and heart have successfully been carried out using SDS; through which ECM remains intact.[28,29]

Among the various types of cells used in tissue engineering,

stem cells have received much attention due to their unique features such as differentiation into various cell types and self-renewal in terms of injured tissue repair.[30] Bone marrow mesenshymal stem cellsis rapidly developed in the culture medium due to their high proliferation potential. In addition, bone marrow mesenchymal stem cells do not stimulate immunological reactions due to the limited expression of MHC Class I antigen and the lack of MHC Class II gene expression. Hence, they have no transplant rejection and therefore are an appropriate option for allogeneic transplantation.[31,32]

### Objectives

The current study focused ondecellularization and demineralization of the bovine femur dense bone as a xenograftbioscaffold for bone tissue engineering studies. The present research aimed to provide a three dimensional (3D) bioscaffold derived niche by demineralization and decellularization of bovine femur dense bone via physical and chemical prototype.It was found that the bovine femur dense bone could be used for the preparation of natural xenograftbioscaffold, which potentially may be applied in the regeneration of bone tissue.

### Materials and Methods

### Preparation of demineralized and decellularized bioscaffold from bovine femur bone

In this step, the bovine femur was immediately prepared after slaughter. Then, the dense bones of bovine femur were cut into 1×1 cmpieces. The samples were washed by physiological serum, and the bone fragments were placed five times in warm water (4 min each time) to completely remove the bone lipid.Afterwards, they were stored at -4° C (Figure-1).[33] Next, after thawing at the room temperature, the bone fragments were washed with phosphate-buffered saline (PBS) several times. In demineralization of dense bone, the bones were placed in 1 N hydrochloric acid (HCL) solution at 37 °C for 30 h, and the solution was replaced with a new one every 6 h. Then, in order to reduce the acid residues, the bone fragments were rinsed with distilled water, and in the next step, these fragments were placed in chloroform/ methanol solution (1:1) (Sigma-Aldrich) for 1 h. After completion of these steps, the bones would become very soft and could be sliced with a razor and then stored at -20 °C. At the decellularization stage, the bone fragments were transferred into liquid nitrogen at -196 °C for 2 min. The

samples were immersed in distilled water for rapid thawing and then were put in PBS for 10 min. This process, which led to the lysis of cells, was carried out five times. At the chemical stage of decellularization, the bone fragments were exposed to 2.5% SDS solution at room temperature for 8 h. Then, in order to complete the decellularization process, it is necessary to reduce residual SDS from the bioscaffolds and sterilize them. For this reason, two-step washing using 75% ethanol and PBS was conducted for 30 min at room temperature.[19,34,35]
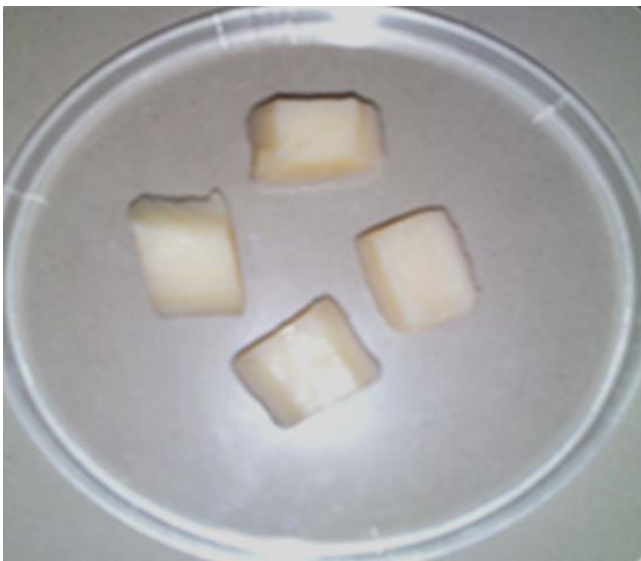


**Figure-1**. Macroscopic image of dense bone fragments prepared from bovine femur bone

## Morphologic study of bioscaffolds by scanning electron microscopy

In order to investigate the surface features of bioscaffolds, Scanning Electron Microscopy (SEM) (LEO1430VP, Germany) was used.Bioscaffolds were first cut into small pieces, covered with gold, and eventually placed in a special place in the device. Finally, the necessary images were recorded.[36]

## Isolation and culture of BM-MSCs

In this stage, Wistar rats were anesthetized by chloroform, and their femur and tibia bones were separated. Next, the two tips of femur and tibia bones were cut with sterile scissors. Afterwards, the total volume of bone marrow was obtained in a Dulbecco's Modified Eagle's Medium (DMEM, Gibco, New York, NY) enriched with 15% fetal bovine serum (FBS, Gibco, Netherlands) and containing 100 µLpenicillin/streptomycin (Gibco) using a syringe. Then, the incubation of cells was carried out at 37° C and 5% $CO_2$. The old culture medium was replaced by a new one every three days until the cells reached 80-90% confluent. After removing the blood and stromal cells, BM-MSCs were purified four times using trypsinization (0.25% trypsin/EDTA solution, Biosera, UK).[37]

## Culture of BM-MSCs on bone-derived bioscaffold

First, the two sides of the bioscaffold were sterilized under UV radiation for 15 min. Afterwards, the bioscaffolds were put in 24-well plates and then washed with PBS.The bioscaffolds derived from the bone were immersed in the DMEM solution for 1 h before the cell culture, and then seeded in 200 µL aliquots containing $2 \times 10^5$ cells. The incubation of bioscaffolds in the medium containing BM-MSCs was carried out in a humidified atmosphere with 5% $CO_2$ at 37 °C for 3 h. Then, more culture medium was poured to fully cover the bioscaffolds. The culture medium was replaced every 3 days, and the samples were studied using the SEM analysis.

## Bioscaffold biocompatibility study

In order to investigate the bioscaffold biocompatibility, BM-MSCs were cultured on the bioscaffold as performed in the previous steps. In the two specified periods (days 1 and 7), the bioscaffolds cultured with cells were transferred to a new 24-well plate. MTT (3- [4, 5-dimethylthialzol-2-yl]-2, 5-diphenyltetrazolium bromide) was added to each well of the cell culture plate. The plates were then placed in an incubator with humidity of 98% and 5% $CO_2$ at 37 °C for 4 h. After 4 h of incubation, the medium on the cells was slowly removed, and 100µL of DMSO solvent (Merck, Germany) was added to each well for solving the purpleformazan crystals obtained from the reduction of MTT. The amount of optical absorbance (OD) of the produced colour in each sample, which is directly related to the number of active metabolic cells, was measured by Elisa reader (URIT-660, China) at a wavelength of 570 nm.[38]

## BM-MSCs differentiation into osteoblast on bioscaffold

This differentiation was done on the bioscaffold using third passage cells. First, the cells were cultured on bioscaffolds in 24-well plates; and in the next day, the culture medium of wells containing bioscaffolds was removed, and after washing the bioscaffold-cell with PBS, the osteoblastic differentiation medium was added. The osteoblastic differentiation medium contained 50 µg/mLascorbic acid 3-phosphate (Sigma-

Aldrich, St. Louis, USA), 10 nmoldexamethason (Sigma-Aldrich, St. Louis, USA), and 10 mmol beta-glycerol (Sigma-Aldrich, St. Louis, USA). The treatment duration was 21 days, and the medium was replaced every two days.[37]

### Histological studies

All control and decellularized bone samples were fixed in formalin, and dehydrated after rinsing in a graded series of ethanol. Then, the paraffinization, sectioning (5μm), deparaffinization by xylene, rehydration, and staining were carried out. Finally, Hematoxylinand Eosin, H & E, (Merck, Germany) staining was used to determine the decellularization process.[39]

### Radiography

Technical radiography is widely used in scanning the internal organs of the body and can also recognize the crystalline structure of objects and thus their constituents. To determine the decalcification rate, the demineralized and control bone samples were individually radiographed. The bone samples were cut in 2 × 2 cm, placed in decalcification solution for 30 h, and then prepared for scanning.[40]

### Calcium oxalate test

Calcium oxalate test was used to detect the presence of calcium in the decalcification solution and to demonstrate the decalcification process. In this test, first, 2 mL of the decalcification solution, i.e. HCL 1N, was alkalized with a few drops of 25% ammonia solution, and the pH of the solution was reached above 7. Then, a few drops of 3% ammonium oxalate solution were dripped into the solution, and the solution was evaluated based on the colour change.[40]

### Evaluation of osteogenic differentiation of BM-MSCs

Alizarin red staining (Sigma-Aldrich) was used to evaluate the osteogenic differentiation of the cells on the bioscaffold and the cells without bioscaffold conditions. Staining was carried out on the cells cultured for 14 and 21 days as follows: After removing the medium and washing the cells with PBS, the washed cells were fixed with 4% formaldehyde for 20 min, and then rewashed with PBS. Finally, 1% alizarin red stain solution was added and incubated for 8 min.After that, washing with distilled water were carried out several times, and then in order to examine the presence of red calcium depositions, the cells were evaluated under the microscope.

The entire staining process for BM-MSCs without a differentiation medium was performed as a control. Moreover, in order to stain the differentiated cells on the bioscaffolds, the cell free bioscaffolds was stained as a control.

### Results

#### Evaluation of demineralized and decellularized bioscaffolds

Evaluation of the demineralization process of dense bone by radiography and calcium oxalate test revealed that the calcium content was 2170 and 230 in the control and demineralized dense bones, respectively. Figure-2 indicates that the calcium content in the control bone was 10 times higher than that of the demineralized bones. Therefore, the decalcification process was effective, and more than 80% of the bone was decalcified (Figure-2). Calcium oxalate test was applied to detect the presence of calcium in the demineralization solution and was repeated every 6 h during the demineralization process that lasted for 30 h. Figure-3A shows that the amount of the calcium decalcified from the sample was high and the solution became completely milky by performing the staining test. Furthermore, with the passage of time and replacement of the decalcification solution, the content of released calcium decreased. In the final step, no colour change was observed by performing a test in decalcification solution (Figure-3D).

It was necessary to replace the solution for each test and wait for 6 h, and then test the fresh solution. The results indicated the complete decalcification.Decellularization of the dense bone by H&E staining showed that the samples treated with 2.5% SDS for 8 (unit) were completely decellularized as compared to the control sample, and the samples treated with 2.5% SDS for 4 h (Figure-4). However, decellularization with 2.5% SDS for 8 h led to complete decellularization and maintenance of the bone ECM structure.

#### Evaluation of the morphology of bioscaffolds by SEM

The electron micrographs obtained from the bioscaffold surface are depicted in Figure-5. The bioscaffold had porous structure. Moreover, the micrographs also showed lack of cell on the bioscaffold surface, and hence confirmed the completion of decellularization process.

#### Extraction of rat bone marrow mesenchymal stem cells

On the third day of culture, the solid residues from the bone marrow were removed during rinsing, and the cells in the colonies were uniformly spread on the bottom of the flask as a result of trypsinization and re-culture. After one week, cell

colonies were recognized again, and the cells were spread by trypsinization. After 24h, the confluency of the cells reached 100%, and the non-adhesive cells including floating blood cells were almost eliminated on the flask medium (Figure-6).
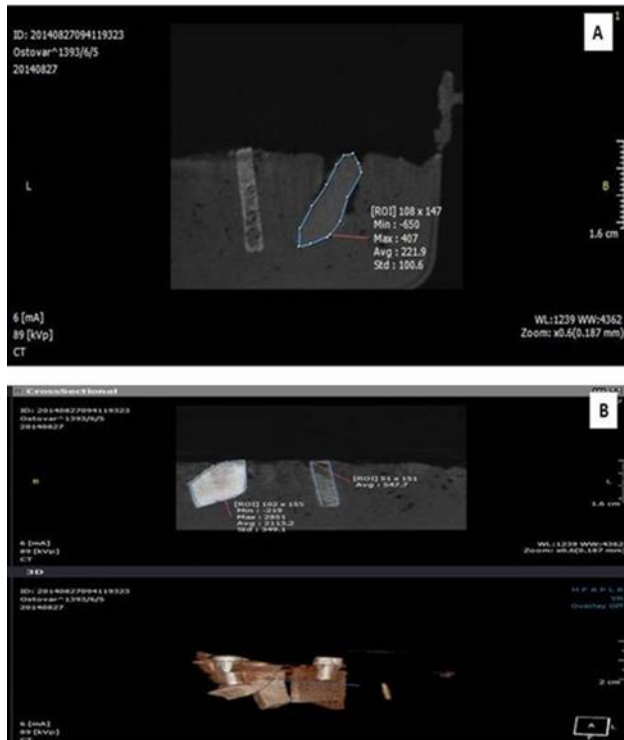


**Figure-2**. Radiography: (A) Demineralized bone and (B) Control bone with the calcium contents of 230 and 2170 (unit), respectively
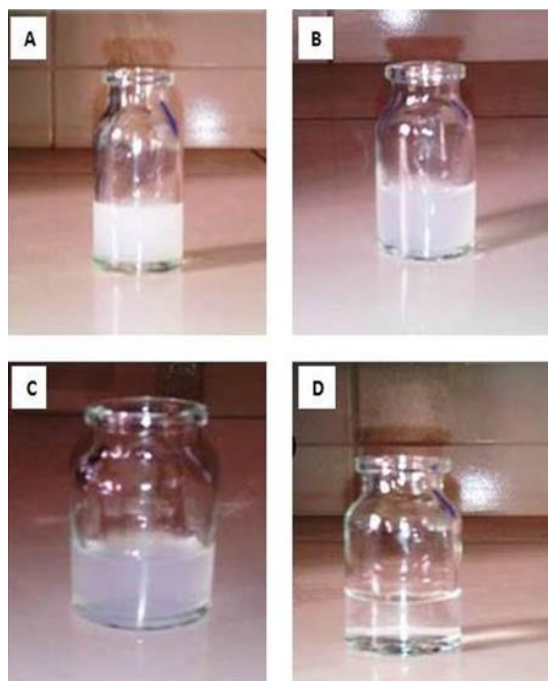


**Figure-3**. Calcium oxalate test for released Calcium contents during (A) 0- 6 (B) 6-12 (C) 12-18(D) 18-24 hours of bone demineralization
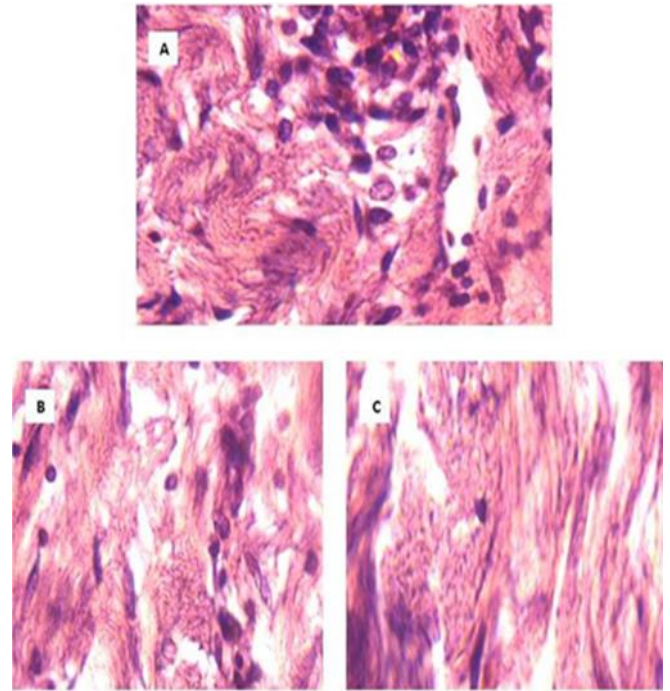


**Figure-4**. H&E staining of dense bone (A) Control staining (B) Staining of bone samples after 4 h (c) Staining of bone samples after 8 h. After treatment with 2.5% SDS for 8 h (40x magnifications), decellularization and preservation of the ECM structure were complete.

## Adherence of BM-MSCs on the bioscaffold

Prepared bioscaffolds using 2.5% SDS for 8 h were the best option for stem cell culture. Figure-7 represents the successful adherence of the cells on the bioscaffold. It seems that decellularization quality may influence the BM-MSCs attachment on the bioscaffold.

## Biocompatibility of bone-derived bioscaffold

The biocompatibility of cells was determined using MTT assay based on the reduction of mitochondrial enzyme activity in BM-MSCs cultured on the bioscaffold. Evaluation of the amount of viable cells on the bone bioscaffold after 1 and 7 days, using Tetrazolium dyeassay with absorbance measurement at the 570 nm, revealed the presence of living stem cells on the bioscaffold (Figure-8). The comparison of the absorbance between control cells and the bioscaffold surface cells indicated that the bioscaffold cells had less absorbance, which was due to the penetration of cells into the bioscaffold over time and lack of tetrazolium dye access to these cells.
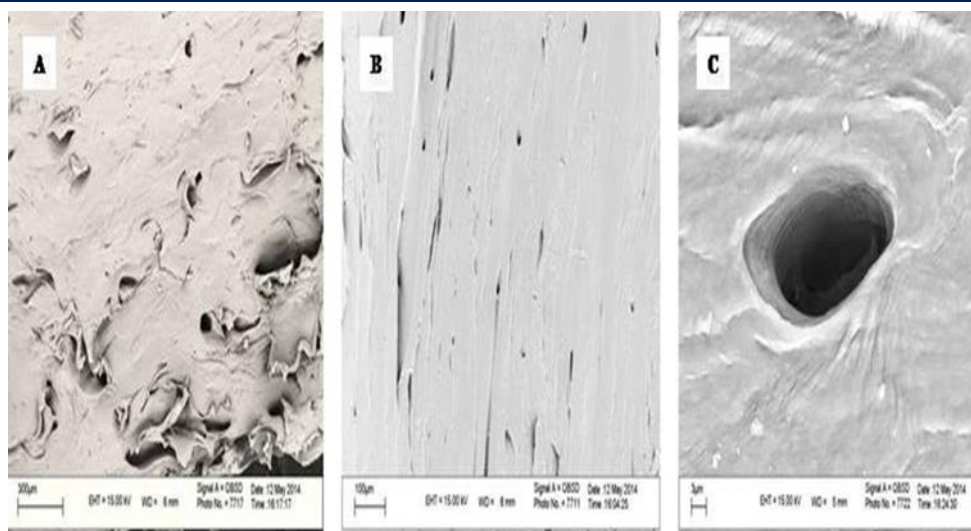
**Figure-5.** SEM images of decellularized dense bone bioscaffold with magnification. (A) 150x (B) 300X (C) 5KX
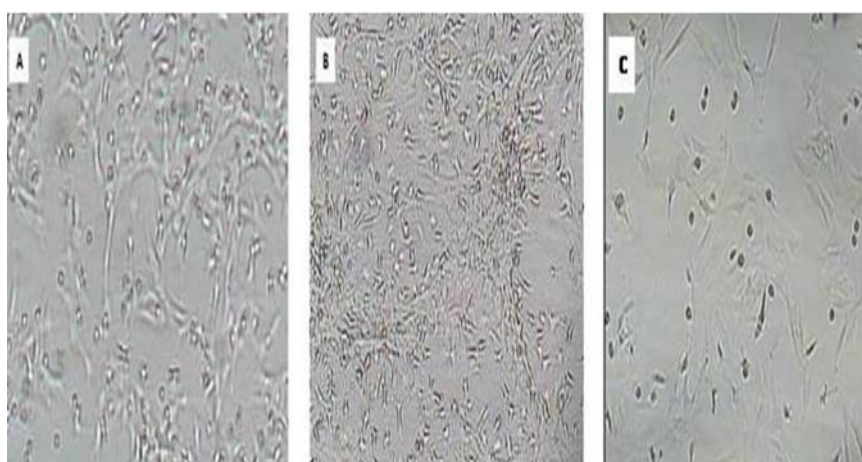


**Figure-6.** Morphology of rat bone marrow mesenchymal stem cells (A) The third day of culture after the first change ofenvironment (B) The fifth day after trypsinization and reconnecting (C) fourth cell passage (40x magnification).
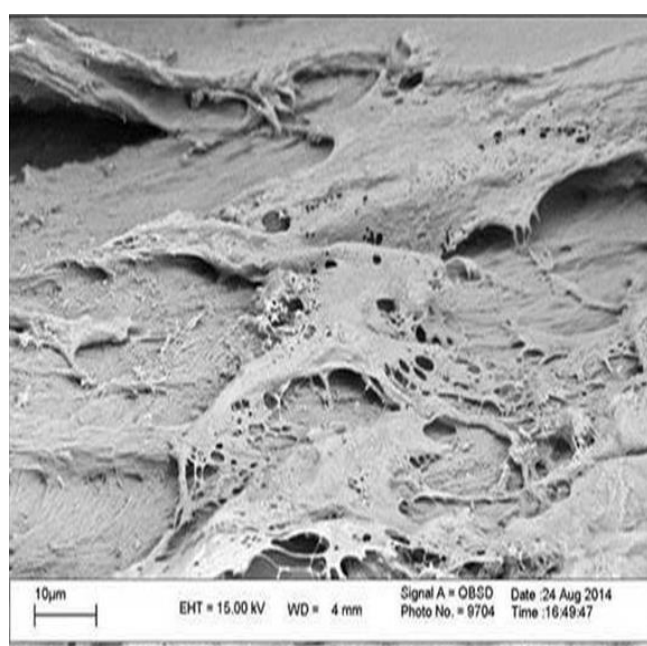


**Figure-7.** Bioscaffold seeded with BM-MSCs. The attached cells are obvious on bone trabeculae.

## Osteoblastic differentiation and calcium deposition

Alizarin red staining is one of the most widely used methods for determining the amount of calcium depositions in osteogenic culture medium. The bioscaffolds attached BM-MSCs exposed to osteogenic differentiation inducing medium for 21 days were evaluated by Alizarin red staining.Calcium deposition on the bioscaffold confirms the osteoblastic differentiation of BM-MSCs. Also, the first signs of morphologic changes and bone differentiation were observed 7 days after differentiation-induction. At the end of the differentiation period (21 days), a large amount of secretion of inorganic matrix was observed. The staining results of the cells treated with differentiation medium on day 21 revealed that the cells were red due to calcium deposition under non-bioscaffold conditions. The control stem cells were not stained at all due to the lack of a differentiated environment, as well as the control bioscaffold without cells. Cell-containing bioscaffolds, which were stained after 21 days of treatment under a differentiated medium, became red in Alizarin red staining due to the deposition of calcium masses (Figure-9)

## Discussion

In bone tissue engineering, the bioscaffold must have osteogenesis conditions for mechanically strength, integration and providing a suitable culture medium for osteoblast cells.[41] Given the current need for different types of bone grafting, autograft is considered the best type of graft. Due to problems such as the limitation of donor, increased and prolonged recovery period, the likelihood of death, experts consider allografts as a suitable alternative for autografts. However, allografts are not able to rapidly stimulate bone growth, but can reduce the length of surgery and the level of pain in patients after surgery.[42] Xenografts have been widely used as human hosts for unlimited access to graft material. The bovine bone is one of the sources of xenografts.[43,44,45] In this research, we tried to demineralize and decellularize the bone using physical and chemical methods. The demineralized and decellularized bone was used as a bioscaffold for culture of BM-MSCs. Bioscaffold characteristics were examined using SEM and histological methods. In this investigation, physical methods, rapid freeze in liquid nitrogen, and chemical methods including SDS were used for decellularization. SDS is a common anionic detergent that damages the membrane, causes tissue decellularization by dissolving the membrane and denaturation of proteins, and reduces the immunogenic response after bioscaffold graft.[21-46] Moreover, hematoxylin-eosin staining was used to confirm cell decellularization. Figure 4 displays that the number of cells was high in the staining of the control sample and decreased by half after 4 h of decellularization. In the final stage, the sample was under 2.5% SDS treatment for 8 h, and the staining revealed that its application for the mentioned time can completely eliminate the cells.

In the production of bone bioscaffolds, the decalcification process is also important. The bones were placed in the hydrochloric acid solution and were softened after the desired time.Extremely fine carbon dioxide bubbles were formed at the surface of the samples decalcified with acid; however, they do not usually bring about adverse effects in microscopic observation.Here in, dense bone decalcification was on temperature and acidity. It was in accordance with a previous study about cortical bone. In other word, as the concentration of acid and temperature increase, the decalcification rate increases, as well.[47] To prove decalcification, radiography and calcium oxalate test were used. The decalcified and control samples were radiographed, which represented decalcification of more than 80% of the samples.Also, the results of decalcification process in this study correspond to those of Castro.[48] Oxalate calcium test was used to prove decalcification and detect the presence of calcium in the decalcification solution. Examination of bioscaffolds by SEM also showed porous surfaces that facilitated the migration of cells and transportation of nutrients during cell culture (Figure-5).

In order to investigate the biocompatibility for the culture of BM-MSCs, 24 h after cell seeding on bioscaffold, evaluation was done using a SEM (Figure-5). The performed evaluations by SEM demonstrated successful cell binding, so that the stem cells completely covered the surface of bone-derived bioscaffolds. MTT measurement also confirmed the SEM results. Moreover, the mentioned results were consistent with those reported by Zhou et al, and indicated that the bone-derived bioscaffolds offered good biocompatibility.[34]
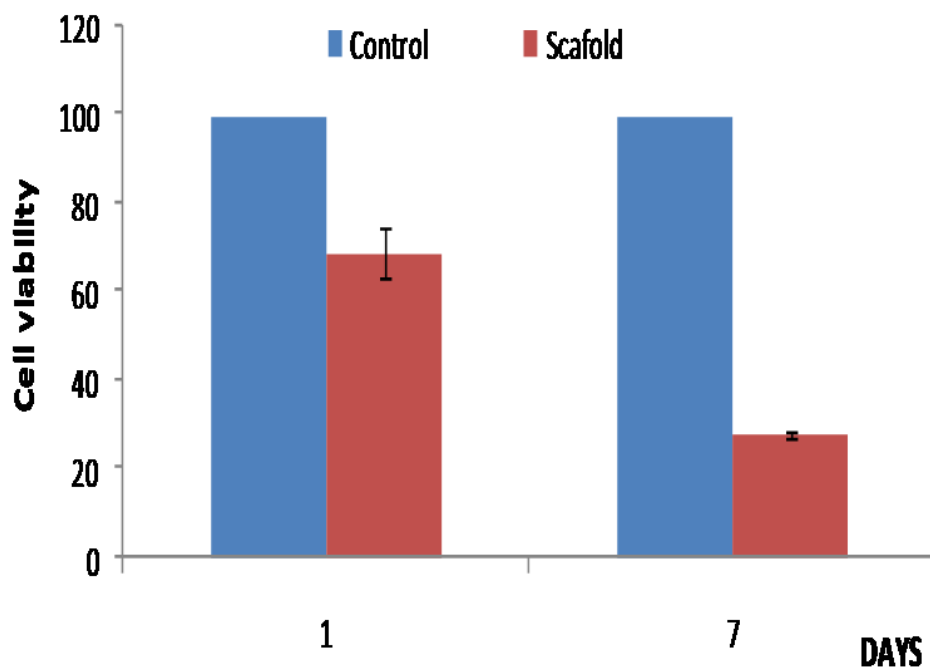
**Figure-8.** Evaluation of BM-MSCs viability on bone bioscaffold according to MTT assay.
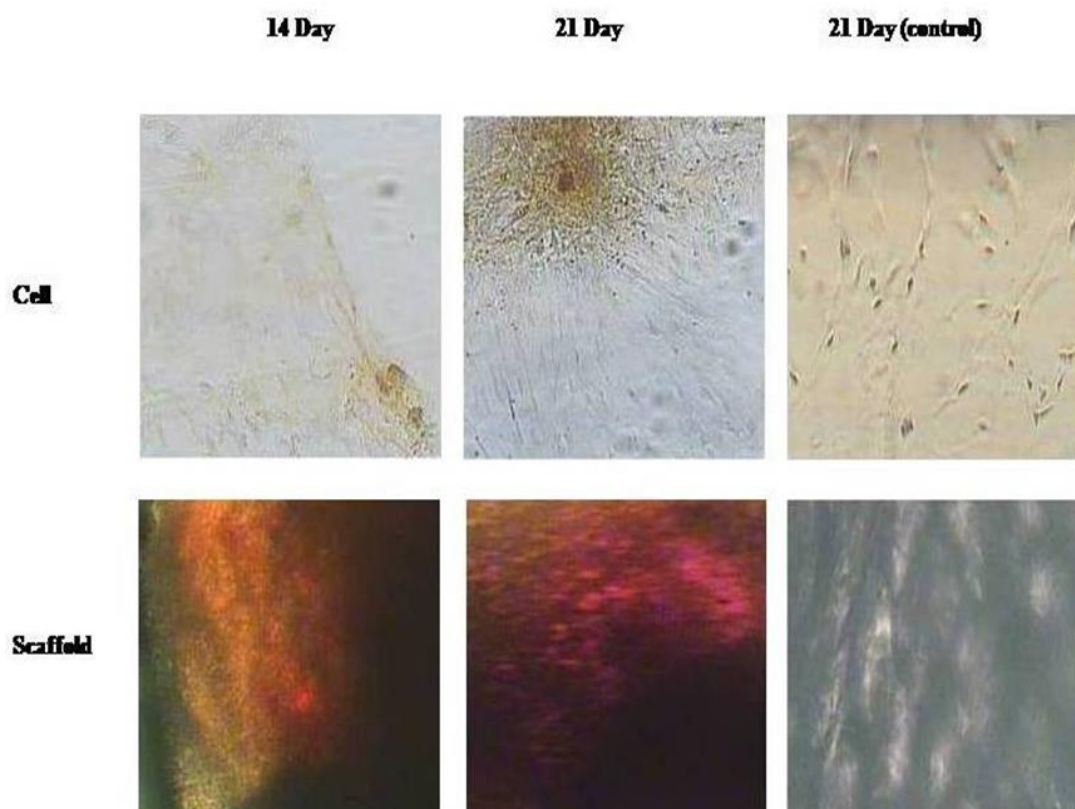


**Figure-9.** The cell images were characterized by differentiation in a two-dimensional environment (control) and a bioscaffold surface prepared by Alizarin red staining method. The red colour indicates the amount of calcium deposition in the cell culture medium.

In this study, the BM-MSCs differentiation process to osteoblasts was explored with and without bioscaffolds. One of the changes that can be assessed in osteoblast differentiated cells is the amount of calcium deposition in these cells. Alizarin red staining, which converts calcium deposition into red, confirmed the osteoblastic

differentiation of cells after 14 and 21 days of treatment in an osteoblastic differentiation medium. These findings suggested that a dense bone-derived bioscaffold obtained by physical and chemical methods can support the junction and maintenance of cells.In this regard, our results confirmed more calcium deposition with bioscaffold in comparison with scaffold free condition. In general, the results revealed that the dense bone of bovine femur can provide a 3D microenvironment for BM-MSCs culturing and differentiation *in vitro.*

## Conclusions

The results of this study showed that the bioscaffold derived from the dense bone of the bovine femur can be used as a bioscaffold in bone tissue engineering. To test the bioscaffold, BM-MSCs were used to be cultured on a fully decellularized bioscaffold to observe the effects of extracellular matrix on these undifferentiated cells. Primary results and histological studies showed that demineralized and decellularized bioscaffolds could be a suitable 3D model for the movement, differentiation, adhesion, and migration of cells. However, the present research was a preliminary study that focused on the osteoporosis process on this bony bioscaffold *in vitro.* Further in vivo studies are recommended regarding bone defects using this bioscaffold.

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## Authors' Contribution

All authors pass the four criteria for authorship contribution based on the International Committee of Medical Journal Editors (ICMJE) recommendations.

## Conflict of Interests

Conflict of interest the authors declare that they have no conflict of interest.

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