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RESEARCH PAPER

Investigation of osteoblast-like cells cultured on nano-hydroxyapatite/chitosan based composite scaffold in the treatment of bone defects and limited mobility

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ABSTRACT

Objective(s): Design and construction of biocompatible and biodegradable scaffolds are among the main goals of tissue engineering. Recently, use of nano-hydroxyapatite as a bioactive bioceramic agent with high similarity to the mineral phase of the human bone tissue, in combination with biodegradable polymers and implant coatings has attracted the attention of researchers in the field of biomaterial sciences. The present study aimed to assess the differentiation of bone marrow stromal cells (BMSCs) in osteoblast-like cells on the chitosan/polyethylene oxide (PEO)/nano-hydroxyapatite scaffold in mature rats.

Materials and Methods: Chitosan and PEO solution with the weight ratio of 80:20 and 70:30 were prepared, and 2% weight of nano-hydroxyapatite was added. Nanofibers were prepared using the electrospinning method, and the morphology was studied using scanning electron microscopy (SEM). Afterwards, the BMSCs of mature rats were cultured on nanofibers and differentiated by adding a differentiation medium. The survival of the differentiated cells was evaluated at the end of the first, second, and third week using acridine orange staining, and the morphology of the differentiated cells exposed to nanofibers was assessed using SEM.

Results: The mean diameter of the nanofibers with the ratio of 80:20 was 150±17 nanometers. The differentiation of BMSCs into the osteoblast-like cells on nanofibers was confirmed using Alizarin red staining. The results indicated a significant decrease in the survival of the differentiated cells in the nanofiber groups by the end of the third week of differentiation compared to the control samples.

Conclusion: According to the results, BMSCs could be differentiated into osteoblast-like cells in the presence of the chitosan/PEO nanofibers containing nano-hydroxyapatite.

Keywords: *Bioceramics, Bone tissue engineering, Chitosan, Nano-hydroxyapatite, Nanocomposites*

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INTRODUCTION

Loss of organs and the ultimate failure of a vital organ due to diseases and injuries are considered to be substantial challenges for healthcare authorities as the substitution of tissues and organs in such patients largely depends on donor compatibility. Conventional replacement procedures (e.g., artificial prosthesis) are not associated with tissue restoration, and their connection with the host tissue may cause problems. In addition, these devices may induce inflammatory responses to the host tissue. The main purpose of tissue engineering is to design and develop similar structures to the natural

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structure of a tissue in the living body in order to repair injuries and tissue lesions [1, 2].

Bone injury is considered to be a significant challenge in medicine, the treatment of which imposes substantial costs on healthcare organizations every year [1, 2]. Bone tissue engineering has three key components, including scaffold, cell, and growth factor. The bone structure is composed of a combination of these components [3]. In tissue engineering, scaffold acts as a temporary matrix for cell proliferation. On the other hand, the porosity of nano-scaffolds allows bone growth inside the scaffold. Nano-scaffolds must have such properties as biocompatibility, biodegradability, non-toxicity, and bio-absorbability [4].

Recently, use of polymeric nanofibers in the production of scaffolds has been on the rise, and reports have been suggestive of the success of this method. Chitosan (CS) is a natural polymer that is used in combination with polyethylene oxide (PEO) in electrospinning, increasing the efficiency of the spinning process in order to obtain uniform fibrous structures without bead. In this process, triton X-100 and dimethyl sulfoxide (DMSO) are added as the surfactant and co-solvent, respectively [5].

Hydroxyapatite is a bioactive material that is widely used in bone repair to improve the biological properties of metal prostheses as coatings in the form of powder or nanoparticle. Bioactivity is defined as the capability of a substance to bind to the living tissues of the body without generating collagen layers. As a result, hydroxyapatite is able to directly bind to the cells in the body and induce bone growth. According to the literature, the main cause of this phenomenon is osteocalcin, which is a protein that could bind to hydroxyapatite. Osteocalcin has a negative charge and forms a bond with the calcium ions that are available in the crystalline structure of hydroxyapatite, thereby acting as a marker for osteoblast cells (bone-synthesizing cells). Furthermore, hydroxyapatite is thermodynamically among calcium- and phosphorus-based ceramics and has the highest stability in the pH and physiological temperature of the human body. The special crystalline structure of this material, which is similar to the mineral phase of the bones and teeth, could be proper substitute to these organs. In other words, hydroxyapatite is the most important bioceramic agent known by humans and currently has numerous applications in biomaterial sciences [6, 7].

Unexpectedly, mesenchymal stem cells are able to reverse under specific circumstances, reverting to other types of cells afterwards. Bone marrow stromal cells (BMSCs) are a type of mesenchymal stem cells that could differentiate to osteoblast-like cells. Therefore, BMSCs are used clinically in the treatment of bone defects and the subsequent movement limitations. Some of the beneficial properties of BMSCs for use in bone tissues include the rapid proliferation in the medium, ease of extraction, causing no immune response, and long-term survival [8, 9].

The present study aimed to assess the differentiation of BMSCs in osteoblast-like cells on the chitosan/PEO/nano-hydroxyapatite scaffold in mature rats.

MATERIALS AND METHODS

Chemicals and reagents

CS with the medium molecular weight of 190 kD, PEO with the molecular weight of 900 kD, and nano-hydroxyapatite with the mean diameter of 150 nanometers were purchased from Sigma-Aldrich (USA). In addition, α-MEM culture medium, glacial acetic acid, gelatin powder, 0.25% trypsin, 0.04% ethylenediamine tetra acetic acid (EDTA), and Triton X-100 were obtained from Merck (Germany). Differentiation medium and dyes were supplied by BIO-IDEA (Iran). Adult Wistar rats were purchased by Baqiyatallah University of Medical Sciences in Tehran, Iran. The animals were randomly divided into equal groups.

Preparation of the CS/PEO solutions for electrospinning

Initially, CS (2% weight/volume) and PEO (3% weight/volume) solutions were prepared separately by dissolution in acetic acid (0.5 M). Following that, the CS and PEO solutions were prepared with the ratios of 80:20 and 70:30, respectively and stirred for five hours using a stirrer. Nano-hydroxyapatite solutions (2 wt%) were obtained from the CS and PEO solutions and sonicated for 20 minutes in order to become uniform [10].

Electrospinning process

Each solution was poured into a two-milliliter syringe using a needle stick (0.5 mm), and the nanofibers were prepared using the electrospinning method. The morphology and mean diameter of the prepared nanofibers were

evaluated using scanning electron microscopy (SEM) [10].

BMSC extraction

BMSCs were extracted from adult Wistar rats (aged 6-8 weeks). Initially, the animals were anesthetized with a mixture of ketamine (50 mg/kg) and xylasein (5 mg/kg). Following that, the posterior organs of the animals were sterilized completely using a surgical betadine solution and 70% ethanol. The thighbone and tibia were cut in half, and the bone marrow was aspirated from the bone canal using a 5-milliliter syringe containing one milliliter of the α-MEM medium [11].

The contents of the syringe were poured in a plate (6 cm) containing the culture medium and 10% fetal bovine serum (FBS) under a biological safety hood and placed in an incubator with 5% CO_2. After 24 hours, the cell culture medium was replaced with a fresh environment. The stromal cells that stuck to the flask, as well as the residual and floating cells, were removed. When the density of the cells stuck to the bottom of the flask reached 80-90%, the cells were passaged (subcultured) using 0.25% trypsin and 0.04% EDTA. The process continued until achieving two passages and the same morphology in the cells.

BMSCs culture and differentiation into osteoblast-like cells on nanofibers

At this stage, 24-well cell culture plates were smeared with 1% gelatin, and the CS/PEO nanofibers (ratios: 70:30 and 80:20, respectively) containing nano-hydroxyapatite (2 wt%) stuck to the bottom of the plate. Following that, the plate was sterilized using 70% ethanol and placed within half a meter from ultraviolet rays for one hour. The cell suspension was obtained from the BMSCs of the second passage in the previous stage. After counting the number in unit volume, the survival of the cells was assessed using the Trypan blue dye. In total, 6×10^4 cells were added to each of cell culture plate and placed in an incubator containing 5% CO_2 at the temperature of 37°C.

Three days after the second passage when cell density reached approximately 90%, differentiation was initiated by adding the differentiating medium containing low-glucose Dulbecco's modified eagle medium (DMEM; 45 ml), dexamethasone (10^{-7} M), ascorbic acid (50 µg/ml), and β-glycerophosphate (10 mM) to target the groups, so that the nanofibers could be completely covered.

Every three days, the previous medium was discarded with utmost precision in order to avoid damage to the membrane and remove the cells, and the new medium was replaced on the nanofibers continuing until day 21. To assess the growth rate, proliferation, and morphology of the living cells, each well was observed at the end of the first and third week using an inverted microscope and photographed using a digital camera attached to the microscope at various magnifications. To count the cells, three microscopic fields with a 40X objective lens were randomly selected, and the cells were counted to calculate the average [12].

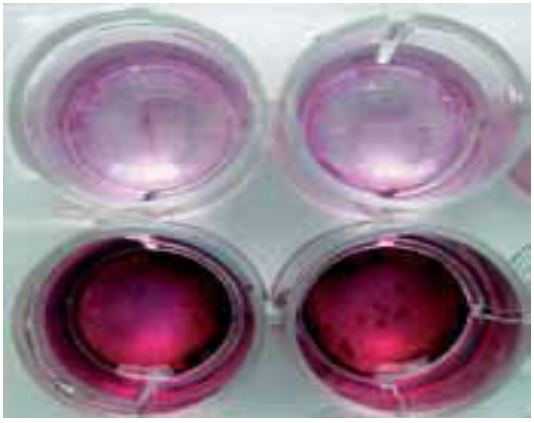

Fig 1. Alizarin red staining; Top: control cells [light and without extracellular calcium phosphate sediments]; Bottom: differentiated cells [darker with extracellular calcium phosphate sediments]

Investigating the process of BMSC differentiation to osteoblast-like cells

Alizarin red staining was used to determine the differentiation. To do so, two grams of Alizarin red powder was completely dissolved in 100 milliliters of distilled water. Afterwards, Ammonium hydroxide (NH_4OH) was added to the solution to adjust the pH at 4.1-4.3. Finally, the brown-black solution was filtered and stored in a dark place [12].

Investigation of cell death

At the end of day 21, acridine orange was used to determine cell death. The cells with an orange nucleus and red cytoplasm were considered as dying cells, and those with a green nucleus and cytoplasm were considered as living cells. Finally, the cells were counted.

Statistical analysis

Data analysis was performed in SPSS version

16. All the experiments were performed in triplicate, and the data were expressed as mean and standard deviation. In addition, the differences were determined by one-way analysis of variance (ANOVA), Dunnett and Duncan post-hoc comparison tests at the significance level of $P<0.05$.

RESULTS

Electrospinning

The optimal conditions for nanofiber formation were obtained by trial and error at the voltage of 19 kV, tip-to-target distance of 11 centimeters, and feed rate of 0.5 ml/h. With increased temperature, the nanofibers formed faster; however, considering the evaporation of the solvent at higher temperatures than room temperature, the optimum temperature conditions were considered ambient for the formation of nanofibers. Moreover, the addition of Triton X-100 and DMSO increased the speed of nanofiber formation.

Electrospinning of CS/PEO solution with the ratio of 70:30 containing nano-hydroxyapatite (2 wt%)

The nanofibers were obtained in the mentioned optimal conditions through electrospinning, and their mean diameter was calculated to be 100±13 nanometers using the ImageJ software.

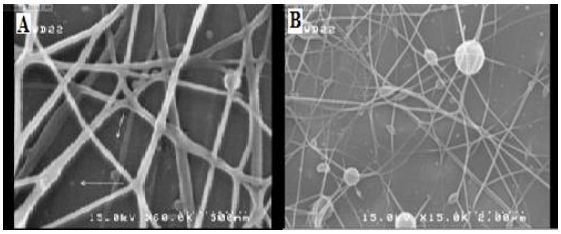

Fig 2. SEM images of CS/PEO nanofibers (70:30) containing nano-hydroxyapatite (2 wt%) at magnification of A) 60,000 X and B) 15,000 X (white arrows show nano-hydroxyapatite with average diameter of 100 nm)

Electrospinning of CS/PEO solution with the ratio of 80:20 containing nano-hydroxyapatite (2 wt%)

Electrospinning was performed in the mentioned optimum conditions. The SEM images revealed the uniform composition of the nano-hydroxyapatite and polymeric solution, the mean diameter of which was calculated to be 150±17 nanometers, showing higher quality compared to the produced nanofibers with the ratio of 70:30.

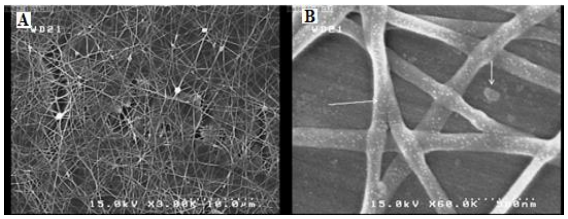

Fig 3. SEM images of CS/PEO nanofibers (80:20) containing nano-hydroxyapatite (2 wt%) on chitosan film at magnifications of A) 3,000 X and B) 60,000 X (white arrows show nano-hydroxyapatite particles)

Fourier transform infrared spectroscopy (FTIR)

In the present study, FTIR was obtained based on the CS and PEO solutions with the ratios of 70:30 and 80:20, respectively containing nano-hydroxyapatite (2 wt%). As is depicted in Fig 4, the peak tensile bonds 3430.16 cm^{-1} (graph A) and 3444.84 cm^{-1} (graph B) represent the OH factor group, and the peak tensile bonds 2108.50 cm^{-1} (graph A) and 2097.35 cm^{-1} (graph B) represent the presence of the carbonate factor group in the solution. In addition, the tensile bonds 1637.56 cm^{-1} (graph A) and 1644.83 cm^{-1} (graph B) represent NO_3^- in the presence of this factor group in the polymeric solution. Bonds 730.24 cm^{-1} (graph A), 717.96 cm^{-1}, and 735.19 cm^{-1} (graph B) also represent the presence of PO_4^{3-} in the solution.

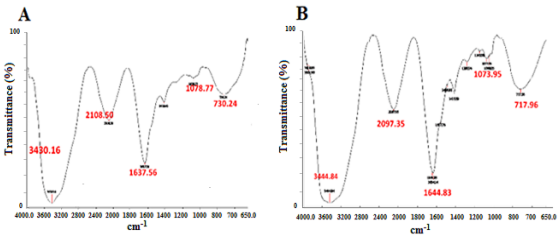

Fig 4. FTIR spectrum of A) CS/PEO polymeric solution (70:30) containing nano-hydroxyapatite (2 wt%) and B) CS/PEO polymeric solution (80:20) containing nano-hydroxyapatite (2 wt%)

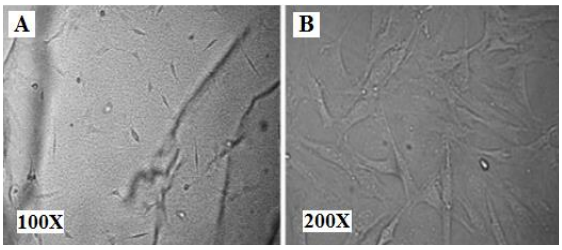

Fig 5. Images of BMSCs cultured on CS/PEO nanofibers (80:20) containing nano-hydroxyapatite (2 wt%) after A) Two days (before onset of differentiation) and B) Four days (at onset of differentiation)

BMSC culture

The onset of the differentiation of BMSCs on day four of cell culturing was resulted from the second passage and observed to be approximately 100% on the produced nanofibers and at the time when the cells reached confluence.

Differentiation of BMSCs into osteoblast-like cells

To investigate the morphology of the differentiated cells after the completion of the differentiation steps and addition of Alizarin red stain, shooting was performed using a digital camera.

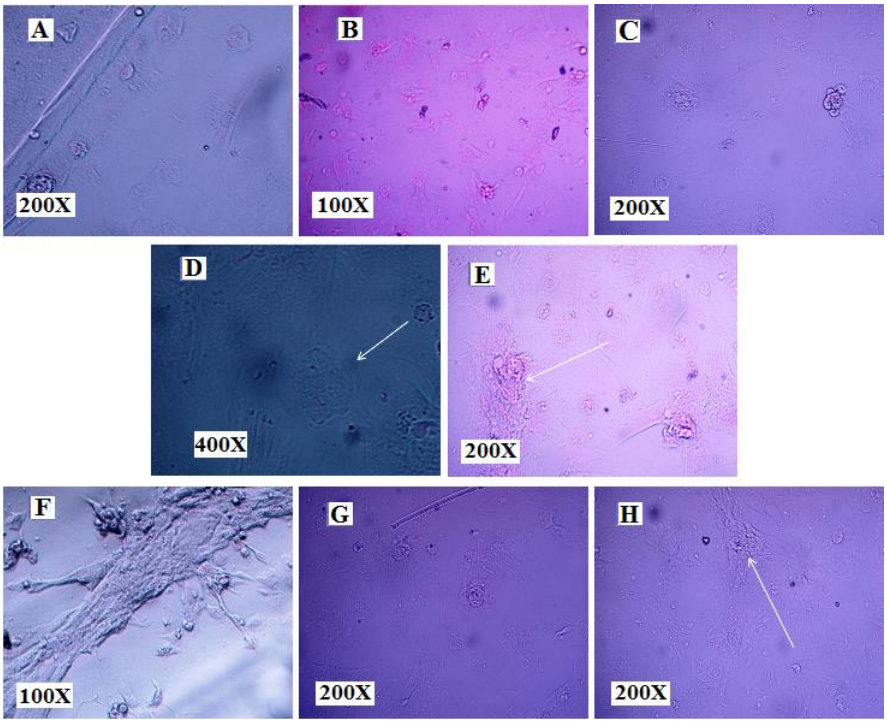

Fig 6. Images of morphological evaluation of differentiated cells on A) CS/PEO nanofibers (70:30) containing nano-hydroxyapatite (2 wt%); B-D) CS/PEO nanofibers (80:20) containing nano-hydroxyapatite (2 wt%); E) Biofilm without nanofibers; F-G) Gelatin control; H) Control (white arrows show accumulation of differentiated cells)

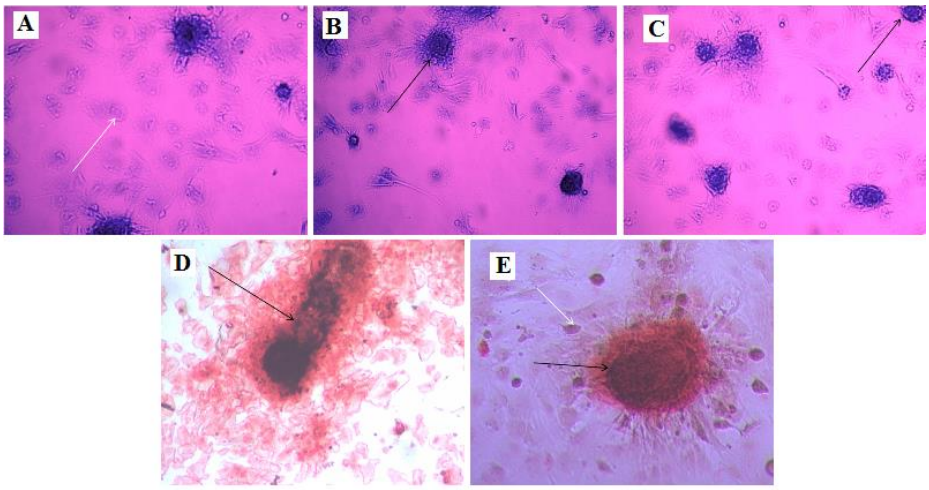

Fig 7. Images of morphological evaluation of differentiated cells on A) CS/PEO nanofibers (70:30) containing nano-hydroxyapatite (2 wt%); B) CS/PEO nanofibers (80:20) containing nano-hydroxyapatite (2 wt%); C) Biofilm without nanofibers; D) Gelatin control; E) Control (black arrows show extracellular calcium phosphate sediments, and white arrows represent osteoblast-like cells [Alizarin red staining, 100X])

The morphological evaluation revealed cellular accumulations and calcium phosphate sediments.

Investigating the differentiation of BMSCs into osteoblast-like cells

After day 21 of differentiation, the cell differentiation process was investigated based on Alizarin red staining. Fig 7 shows the differentiated cells in light red color and calcium phosphate sediments in black masses. During staining, the nanofibers often absorbed the Alizarin red color, which made it difficult to photograph the cells.

Viability analysis of the osteoblast-like cells differentiated from BMSCs

In the acridine orange staining, dying cells were observed with a green nucleus and red cytoplasm, and living cells were detected with a green nucleus and cytoplasm. As can be seen in Fig 8, almost all the cells were alive. Fig 9 depicts the amount of the living cells during the differentiation process.

Results of SEM

At the end of weeks 1-3, the samples were dried after fixing at room temperature and prepared for SEM.

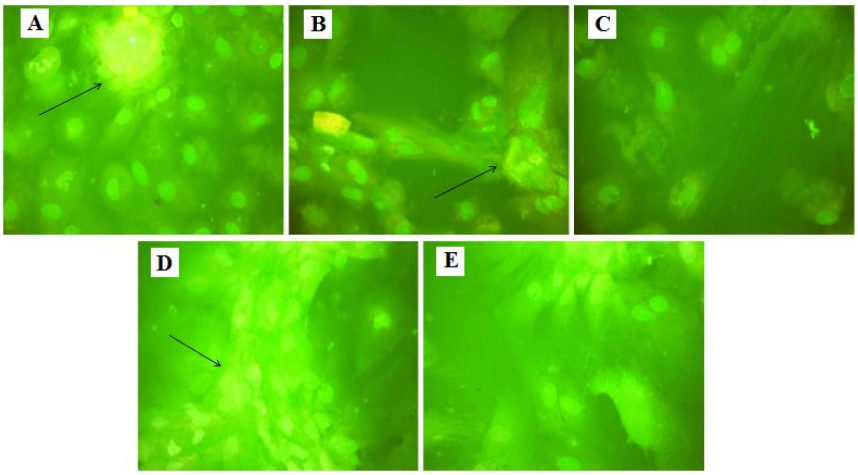

Fig 8. Images of differentiated cells on A) CS/PEO nanofibers (70:30) containing nano-hydroxyapatite (2 wt%); B) CS/PEO nanofibers (80:20) containing nano-hydroxyapatite (2 wt%); C) Biofilm without nanofibers; D) Gelatin control; E) Control (black arrows represent accumulation of differentiated and live cells with green nucleus; acridine orange staining; 400X)

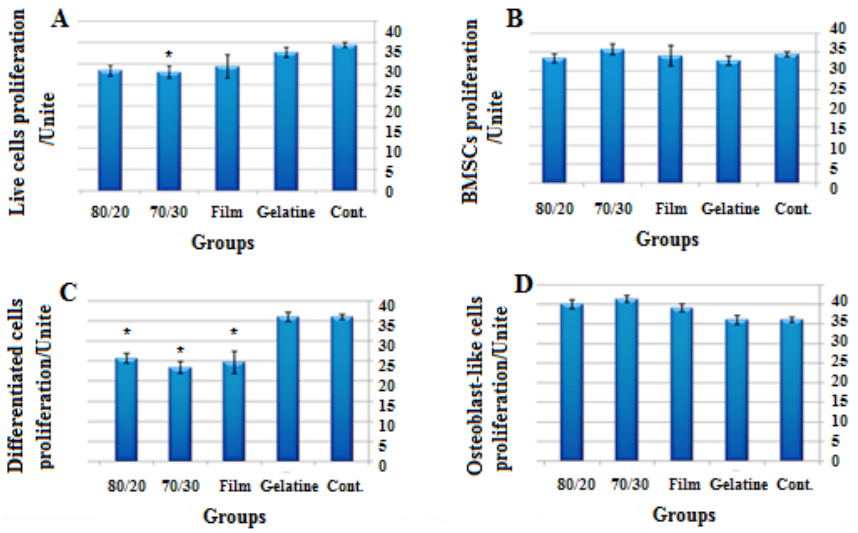

Fig 9. A) Number of live cells in nanofibers and control groups at onset of differentiation (per unit area); B) Number of BMSC cells in nanofibers and control groups at onset of differentiation (per unit area); C) Number of differentiated cells at the end of third week in nanofibers (per unit area); D) Number of live differentiated cells at the end of third week in nanofibers; *Significant difference with control group

The quality of the SEM images of the samples was higher at the end of the first week compared to the second and third week. The poor quality could be due to the duration of nanofiber placement on the differentiation medium and mineralization of the film surface.

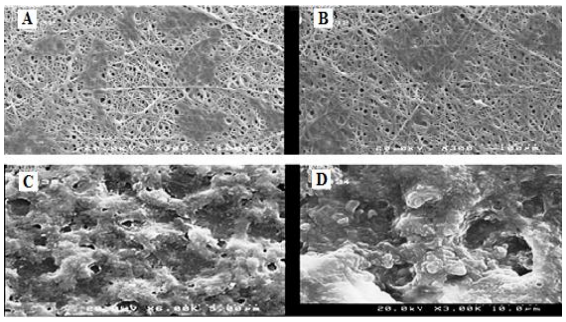

Fig 10. SEM images of BMSCs at the end of first week of differentiation on A) CS/PEO nanofibers (70:30) containing nano-hydroxyapatite (2 wt%; 30,000X; B) BMSCs at the end of first week of differentiation on CS/PEO nanofibers (80:20) containing nano-hydroxyapatite (2 wt%; 30,000X; C) BMSCs at the end of third week of differentiation on CS/PEO nanofibers (70:30) containing nano-hydroxyapatite (2 wt%; 6,000X; D) BMSCs at the end of third week of differentiation on CS/PEO nanofibers (80:20) containing nano-hydroxyapatite (2 wt%; 3,000X)

DISCUSSION

According to the results of the present study, the BMSC cells could easily grow, replicate, and be differentiated onto osteoblast-like cells on the CS/PEO nanocomposite scaffold containing nano-hydroxyapatite.The scaffold used in tissue engineering should be similar to the extracellular matrix of the desired tissue. The CS/PEO nanocomposites that contain nano-hydroxyapatite are optimal for this purpose as they provide the features of a suitable scaffold in terms of mechanical and cellular connectivity. Furthermore, the use of hydroxyapatite particles on the nanoscale is considerably similar to natural scaffolds [13].

A remarkable strength of the current research was the use of mesenchymal stem cells since these cells are highly regarded for clinical applications owing to the absence of immune system stimulation, as well as proper differentiation and proliferation capabilities [8].

In a study conducted by Sheikh *et al.* (2010), the combination of polyvinyl alcohol (PVA) nanofibers and nano-hydroxyapatite was used to generate bone scaffolds, the outcomes of which were considered favorable [14]. In addition, Shahrooz *et al.* produced nanocomposites using polycaprolactone (PCL)/CS/PVA and nano-hydroxyapatite polymers by conducting electrospinning, which was simultaneously led using two nanofiber syringes [15]. However, the mentioned studies were solely focused on providing scaffolds, while in the present study, the scaffold was constructed simultaneous with the investigation of its cellular compatibility. The obtained results indicated the biocompatibility of the nano-scaffold with the cells, as well as the better combination of the hydroxyapatite nanoparticles with the desired polymer.

In another research in this regard, Venugopal *et al.* (2008) developed a scaffold for bone tissue using collagen and hydroxyapatite nanofibers. The results of Alizarin red staining in the mentioned study showed the mineralization of the extracellular calcium phosphate in the medium [16]. However, a major limitation in the mentioned study was cell deficiency due to inadequate proliferation in the scaffold.

Ramay *et al.* (2004) developed a porous calcium phosphate scaffold using nano-hydroxyapatite based on the mechanical properties of scaffolds, such as porosity, which is an important condition in bone tissue engineering. Due to the absence of a polymer in the mentioned research, the produced scaffolds had unfavorable mechanical conditions [17]. On the other hand, Liuyun *et al.* (2009) produced scaffolds for bone tissue engineering using nano-hydroxyapatite/CS and carboxymethyl cellulose. In this scaffold, only hydroxyapatite was used in the nano-size, and the applied polymers had no nanofiber dimensions or forms. Nevertheless, the produced scaffold was compatible with the osteoblast cells. The advantage of the current research over the aforementioned studies was the construction of a nano-scaffold that could act as an extracellular matrix, thereby resulting in the better connection of the osteoblasts to the scaffold and absorbing the regenerative cells into the scaffold by producing cytokines [18].

Another study in this regard was conducted by Emamgholi *et al.* on the cultivation of BMSCs on CS/PEO/nano-hydroxyapatite nanofibers, which resulted in the production of an appropriate coating of cell nano-scaffold to be used for fractures and partial bone fragments. This is consistent with the results of the present study in this regard [10].

The study performed by Rahimi *et al.* regarding

the cultivation of BMSCs on CS nanofiber/film scaffolds and CS/PEO scaffold indicated the biocompatibility and non-toxicity of the produced scaffolds and proper cell proliferation on the mentioned media, which is in line with the results of the present study [19].

Cao *et al.* performed an X-ray study of the transplantation of BMSCs with a tricalcium phosphate (TCP) scaffold in a goat with osteoporosis and condyle femoral bone defects. According to the findings, the formation of a new bone and repair rate were more significant in the group with cultured stem cells on TCP. In addition, the generated bone was reported to be well-integrated with the surrounding tissues of the defected bone. Therefore, the index and rate of the formation of the new bone were reported to be higher in the cell therapy group compared to the other groups. This finding is in congruence with the results of the present study in terms of cell growth and cell proliferation, as well as the bioactivity of the produced scaffold [20].

In a study by Bhattarai *et al.* (2005), various concentrations of the composite solutions of CS and PEO were electrospun. According to the obtained results, the structure of the CS/PEO nanofiber was preserved in water, causing the chondrocytes and osteoblast cells to better bind to the scaffold. Our findings regarding the synthesis of nanofibers and cell culture are consistent with the mentioned study [5].

In another research, Zhou used hydroxyapatite nanoparticles in tissue engineering, reporting their superior compatibility with soft tissues (e.g., skin, muscles, and gum), which make it an ideal candidate for orthopedic and dental implants. Furthermore, hydroxyapatite nanoparticles could be applied to repair hard tissues, increase bone formation, and fill the areas of bone defects, which is in line with the findings of the current research [21].

The study by Tripathi *et al.* was focused on the physico-mechanical and biological evaluation of porous hydroxyapatite scaffold in bone tissue engineering, and the obtained results indicated that osteoblast-like cells were able to bind, multiply, and migrate to the pores of the scaffold. This is in line with the current research [22].

According to the findings of Leukers regarding the preparation of the hydroxyapatite scaffold using the 3-D printing technique for tissue engineering, the cultured MC3T3-E1 cells deeply penetrated into the pores of the hydroxyapatite scaffold and began proliferation. Moreover, the author proposed further investigations on mesenchymal stem cells; the results of the mentioned study are consistent with our findings in terms of biocompatibility, non-toxicity, and cell proliferation [23].

Another study in this regard was conducted by Guan on collagen/hydroxyapatite porous scaffold *in-vitro* using human BMSCs (hBMSCs). The obtained results demonstrated that the use of hydroxyapatite crystals on the scaffold not only increased the cell adhesion and proliferation of hBMSCs, but it could also stimulate osteogenic differentiation. Furthermore, the *in-vivo* results indicated that due to the similarity of the crystallographic structure and chemical components to natural bones, the scaffold is stronger compared to collagen fiber porous scaffolds. Use of the collagen/hydroxyapatite porous scaffold for eight weeks was also reported to stimulate the production of new bones in rats. In the mentioned study, it was proposed that the scaffold could be applied in the treatment of osteoporosis owing to its favorable porosity and excellent compatibility, which is consistent with the results of the present study [24].

CONCLUSION

Limited bone resources in autograft transplantation and concerns about the transmission of pathologic agents have encouraged the production of alternative materials with osteoconductive and osteoinductive properties. Nano-scaffolds are used as biomedical bone substitutes. In tissue engineering, hydroxyapatite is the first option for the repair of bone defects as a biochemical agent with similar formula to the bone mineral phase.

The results of the present study indicated the growth, proliferation, and viability of BMSCs and their differentiation to osteoblast-like cells on the prepared nanofibers. Therefore, it could be concluded that the scaffold of cell nanofibers could be applied in the treatment of fractures and bone defects. In addition, the obtained results suggested that a sample with nano-size dimension could also stimulate osteogenesis.

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Ethical statement

The study protocol was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.MSP.REC.1397.437).

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