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RESEARCH PAPER

Perspectives of chitosan nanofiber/film scaffolds with bone marrow stromal cells in tissue engineering and wound dressing

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ABSTRACT

Objective (s): Several methods have been proposed for repairing defects and damages, one of which is cell therapy. Bone marrow stromal cells seem to be suitable for this purpose. On the other hand, many biometric materials are used to improve and correct the defects in the body. Nanofibers are widely used in the medical industry, especially in tissue engineering, as scaffolds in wound healing and wound dressing. Chitosan/polyethylene oxide nanofibers can be a suitable replacement for routine wound coverages. Hence, this study was conducted to present a combination of these methods.

Materials and Methods: Chitosan/polyethylene oxide nanofibers and thin films of chitosan were produced and optimized by electron microscopy, on which the bone marrow stromal cells were then cultivated. Interactions between the cells and these biomaterials were investigated through viability, morphology, immunocytochemistry and electron microscopy of cells after 6 days. All data were analyzed using Student's t-test and one-way ANOVA tests in SPSS version 16. $P<0.05$ was considered significant.

Results: It seems that the high viscosity of chitosan prevents the formation of nanofibers, while chitosan/polyethylene oxide solutions with 80/20 and 90/10 ratios produce perfect, regular, bead free and non-toxic nanofibers with average diameter of 240±10 and 220±10 nm, respectively.

The results of immunocytochemistry and viability showed that the cells had relatively high proliferation on the thin chitosan membranes, while the results of the electron microscopy showed that the morphology of cells was better on the nanofibers than on the thin membrane of chitosan.

Conclusion: Since bone marrow stromal cells were grown well on chitosan-nanofibers, each of them alone was used in the therapeutic methods. It is better to consider a combination of two methods as the treatment method, especially in tissue engineering and cell therapy.

Keywords: *Biocompatible materials, Cell- and Tissue-based therapy, Nanocomposites, Nanostructures, Tissue regeneration*

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INTRODUCTION

Chitosan (CS), a copolymer of glucosamine and N-acetyl glucosamine units linked by 1-4 glucosidal bonds, is a cationic polysaccharide obtained by the alkaline deacetylation of chitin [1]. CS has been used frequently in many industries due to its natural origin and exceptional properties such as biodegradability, biocompatibility, and non-toxicity. Among them, biomedical applications including tissue-engineering scaffolds and wound healing dressings have attracted a lot of attention lately [2].

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Films and nanofiber membranes exhibit an enhanced efficiency because of their large specific area. Such individual layers can be combined with barrier and structural films to provide the required permeability and mechanical properties, respectively. A number of different methods have been used to obtain nanofibers. More recently, electrospinning has been developed as a novel technique to generate polymeric fibers of nanometric size [3]. Electrospun nanofibers with varied morphology have been obtained by proper selection of system and process parameters such as solvent, polymer concentration, and flow rate [4]. Addition of a second component can facilitate the electrospinning process. For example, it is difficult for fibroin and collagen to be electrospun from their aqueous solutions; however, addition of polyethylene oxide (PEO) can improve the processing ability of these polymers [5]. That the organic solvents can be replaced by aqueous solutions also reduces the potential toxicity and enhances the biocompatibility of the nanofiber membranes for biomedical applications [6].

Adult stem cell populations have been found in many tissues of the human body.

They are believed to be important to the repair mechanism intrinsic to many tissues and organs [7, 8]. The mechanism of compromised wound healing is multifactorial and includes injured stimulatory cell migration to the wound, reduced growth factor production, and tissue remodeling [9]. Bone marrow stromal cells (BMSCs) are one of the adult stem cells that are capable of producing a variety of cytokines and hematopoietic growth factors such as transforming growth factor beta-1 (TGF-1), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) platelet-derived growth factor (PDGF), keratinocyte growth factor (KGF), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF) constitutively, which are very useful for wound healing and defect repair [10].

Compared to the untreated wounds, the wounds treated with BMSCs are healed faster. In each case, BMSCs alone have been used for the treatment of damaged skin, bones, and injuries. One study showed that bone strength was higher when nanofiber/BMSCs were used [11]. It was also shown that the BMSCs associated with PLGA nanofiber scaffold might be useful for improving the functional peripheral nerve repair [12].

On the other hand, use of biological materials such as CS and various technologies such as electrospinning for production of nanofiber as wound coverage and defect dressing is of interest to researchers. Therefore, the aim of the present study was providing CS film/nanofiber scaffolds by BMSCs transplantation in tissue engineering for wound and bone healing [13].

A novel natural wound coverage was prepared by electrospinning of mixed solutions of CS and PEO. It can be an ideal choice for wound dressing that reasonably facilitates wound healing process.

MATERIALS AND METHODS

Chemicals and reagents

Medium molecular weight CS (Mw=1.095×10^6 g/Mol, 85% of deacetylation), PEO (Mw=9×10^5 g/Mol) and low molecular weight CS (75-85% of deacetylation) were obtained from Sigma-Aldrich. The α-MEM culture medium, trypsin 0.25%, EDTA 0.04%, acetic acid and Triton X-100 were provided by Merck. Paraformaldehyde was purchased from Invitrogen. Mouse anti-fibronectin monoclonal antibody and secondary antibody (anti-mouse DAB) were supplied by Chemi-Con. Mouse monoclonal anti-CD44 and anti-CD45 antibodies were obtained from Santa-Cruz Biotechnology.

Preparation of CS/PEO solution

First, 2% CS and 3% PEO solutions were prepared separately by dissolving CS or PEO in 0.5 M acetic acid. The CS and PEO solutions of different proportions were then mixed to obtain mixtures with CS/PEO weight ratios of 100/0, 90/10, and 80/20, and the resultant mixtures were stirred for 24 h. The solutions containing 0–0.5 wt% of Triton X-100 and 0–10 wt% of dimethyl sulfoxide (DMSO) were mixed with CS/PEO solutions, and the mixtures were stirred overnight and centrifuged to remove the air bubbles before use.

Preparation of chitosan thin film (CTF)

For preparation of each membrane using the method described by Cheng [13], the low molecular weight CS with a ratio of 1 wt% was added to double-distilled water at 40°C. Then, 0.5 mL glacial acetic acid was added and the mixture was heated for 5 hours on the magnetic stirrer and was perfectly stirred up. Then, to remove the air bubbles inside the solution, it was centrifuged twice at 2500 rpm for 10 min (Sigma 3-18K).

Next, 7.5 mL of the obtained solution was poured into a 75 mm-diameter Petri-dish and

exposed to 25°C temperature for 24 hours to evaporate. Evaporation of the solvent resulted in formation of the membrane, which was washed with double-distilled water twice and dried at 24°C.

Electrospinning process and analysis of prepared nanofibers

The CS/PEO solution for electrospinning (Farasan Co, Iran) was fed into a 5 mL syringe fitted with a pipette tip of 0.5 mm in diameter. The solution feed was driven by the gravity and the feed speed was controlled by the tilt angle of the syringe. The electrospinning method used in this study was the same method reported by Bhattarai [14]. A DC voltage of 20–25 kV was applied between the syringe tip and a cylindrical collector covered with an aluminum foil and CTF. The 7 cm-diameter cylinder was driven by a DC motor with controllable speed. The typical distance between the syringe tip and the grounded collector was 12–15 cm.

All the spinning experiments were performed at 24°C. The as-spun nanofibers were dried at room temperature. Consequently, nanofibers 90/10 and 80/20 were prepared on the CTF and aluminum foil. Electrospun nanofibers were sputter-coated with Au/Pd, and the morphology of the nanofiber was examined by an SEM (LEO -1455 VP). The mean diameter of the prepared nanofibers was determined by measuring the diameters of the nanofibers at 100 different points in a 645×484, SEM image. The diameters were expressed as mean ± standard deviation.

The prepared nanofibers were characterized by fourier transform infrared spectroscopy (FTIR).

Bone marrow stromal cells isolation

BMSCs were collected from the tibias and the femurs of adult Wistar rats aged 6–8 weeks. The proximal and distal ends of the bones were removed under aseptic conditions, and the bone marrow was aspirated with 5 mL α-MEM containing 500 units of heparin using a 21G needle. The cell pellet was obtained and suspended in α-MEM containing 15% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 25 ng/ mL amphotericin-B. The harvested cells were seeded on a 75-cm^2 flask at 37°C, 5% CO_2 incubator (24 hours). The flasks were washed with PBS in order to remove the hematopoietic cells. The enhanced cells were incubated for 2–3 days until they reached the confluence. The cells were then removed with 0.25% trypsin and 0.04% EDTA for 5–10 min in each passage, and the culture was repeated for three passages in order to obtain a single-cell suspension. The method used to obtain the BMSCs in this study was similar to what had been reported previously [15].

Cell culture and adhesion

Nanofiber 90/10, nanofiber 80/20, CTF, nanofiber 90/10 on CTF, and nanofiber 80/20 on CTF were deposited on 24-well plates and washed several times with 75% ethanol to sterilize. They were then washed with double-distilled water and PBS at neutral pH to remove the residual solvent and surfactants introduced during electrospinning. Next, 10^5 of BMSCs were added to the culture medium containing 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin, were seeded onto 24-well plates, and were put in 37°C, 5% CO_2 incubator afterwards.

Cell proliferation, viability and morphology
Proliferation

To evaluate the growth and proliferation of BMSCs, all photos were taken at different magnifications of each of the houses on the second, fourth, and sixth days by a digital camera (INFINITY1) attached to an inverted microscope (Leica). For cell counting, five microscopic fields with objective lens 20X were randomly selected, and the number of cells/field was counted and recorded.

Viability assay

Trypan blue staining was used to determine the number of the viable cells present in the cell suspension. The intact cellular membrane of the living cells excludes certain dyes such as trypan blue, whereas, the dead cells are stained with dyes due to the disrupted membrane. Thus, the cell suspension was mixed with trypan blue and examined by light microscopy to determine whether the cells absorbed or excluded the dye. In this protocol, the viable cells demonstrate a clear cytoplasm, whereas, the dead cells have a blue cytoplasm.

Morphology

To study cell morphology by SEM, the samples were fixed and washed three times with PBS and once with double-distilled water and dehydrated in

an ethanol series (25%, 50%, 75%, 95% and 100%). The samples were then dried using a critical point dryer and were coated with Au/Pd.

Immunostaining

The BMSCs have the potential to differentiate into various cells and are capable of transdifferentiation into other cells but they do not need differentiation. For this purpose, fibronectin, CD44 and CD45 antibodies were used as described below: The BMSCs were washed in PBS and fixed with 4% paraformaldehyde in PBS for 15 min. The fixed cells were washed twice with PBS before staining. To permeabilize the samples and block nonspecific antigen reactions, the slides were incubated in the blocking buffer for an hour. The buffer consisted of 0.1% Triton X-100 and 10% goat serum in PBS. Then, the slides were incubated in primary antibodies, including mouse anti-fibronectin antibody (1:100), mouse anti-CD45 antibody (1:300), and mouse anti-CD44 antibody (1:300) overnight at 4˚C and washed three times with PBS. The slides were then incubated with the relevant secondary antibody (anti-mouse DAB) for 2 hours at 24°C and washed in PBS twice. Then, they were washed in PBS and examined using a microscope at 100X and 200X magnifications. For negative controls, the primary antibodies were omitted and the same staining procedure was conducted as above. The immunocytochemistry method used in this study was similar to what had been reported previously [15].

Statistical Analysis

All data were analyzed using Student's t-test and one-way ANOVA tests in SPSS version 16.

A significant level of 0.05 was predetermined for all statistical analyses. All data are expressed as mean ± standard deviation.

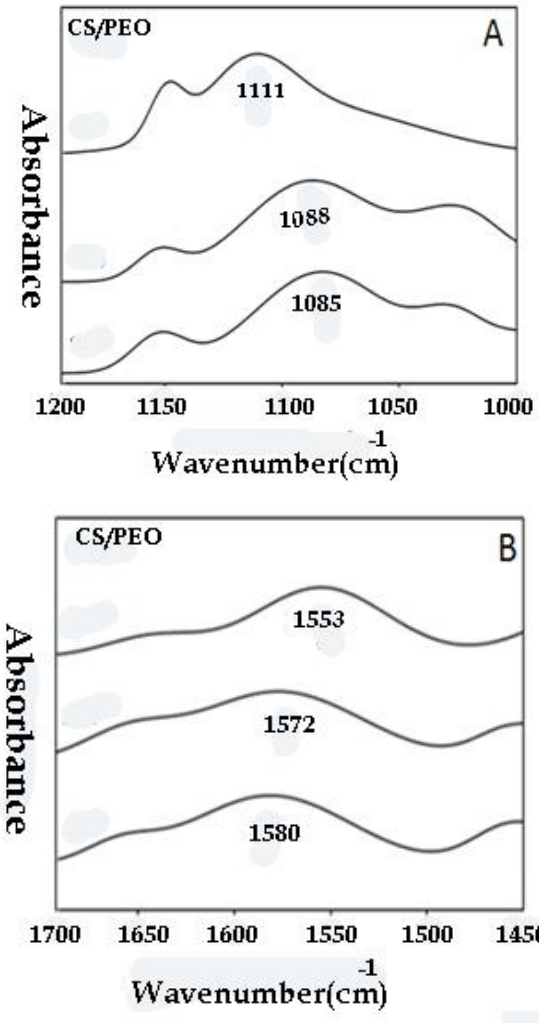

Fig 1. (A) Normalized transmission FTIR spectra recorded at room temperature in the ether (C-O-C) region for neat PEO film and as-spun CS/PEO nanofibers; (B) Normalized transmission FTIR spectra recorded at room temperature in the amine (NH2) region for neat CS and as-spun CS/PEO nanofibers

RESULTS

Evaluation of CS/PEO nanofibers

PEO was introduced in this study to reduce the viscosity of CS solution by interacting with CS through hydrogen bonding, rendering the solution spinnable at higher polymer concentrations. Hydroxyl (OH), carbonyl (C=O-NHR), amine (NH2), and other groups of CS form intra/inter-chain hydrogen bonds [15].

As Fig 1A shows the FTIR spectra obtained for neat PEO and CS/PEO blend nanofibers at various CS/PEO contents. The absorption peak observed at 1111 cm^{-1} in 100/0 of CS/PEO, at 1088 cm^{-1} in 90/10 of CS/PEO, and at 1085 cm^{-1} in 80/20 of CS/PEO is typical of the vibration stretching of the ether (C-O-C) group.

This peak indicated by an arrow, gradually shifts to lower wave numbers by increasing the CS content in the nanofiber.

The FTIR spectra obtained for neat CS and CS/PEO blend nanofibers at various CS/PEO contents in the amine (NH2) stretching region are shown in Fig 1B. The strong peak observed at 1553 cm^{-1} is attributed to the amine band in CS at 1572 cm^{-1} in 90/10 CS/PEO and at 1580 cm^{-1} in 80/20 CS/

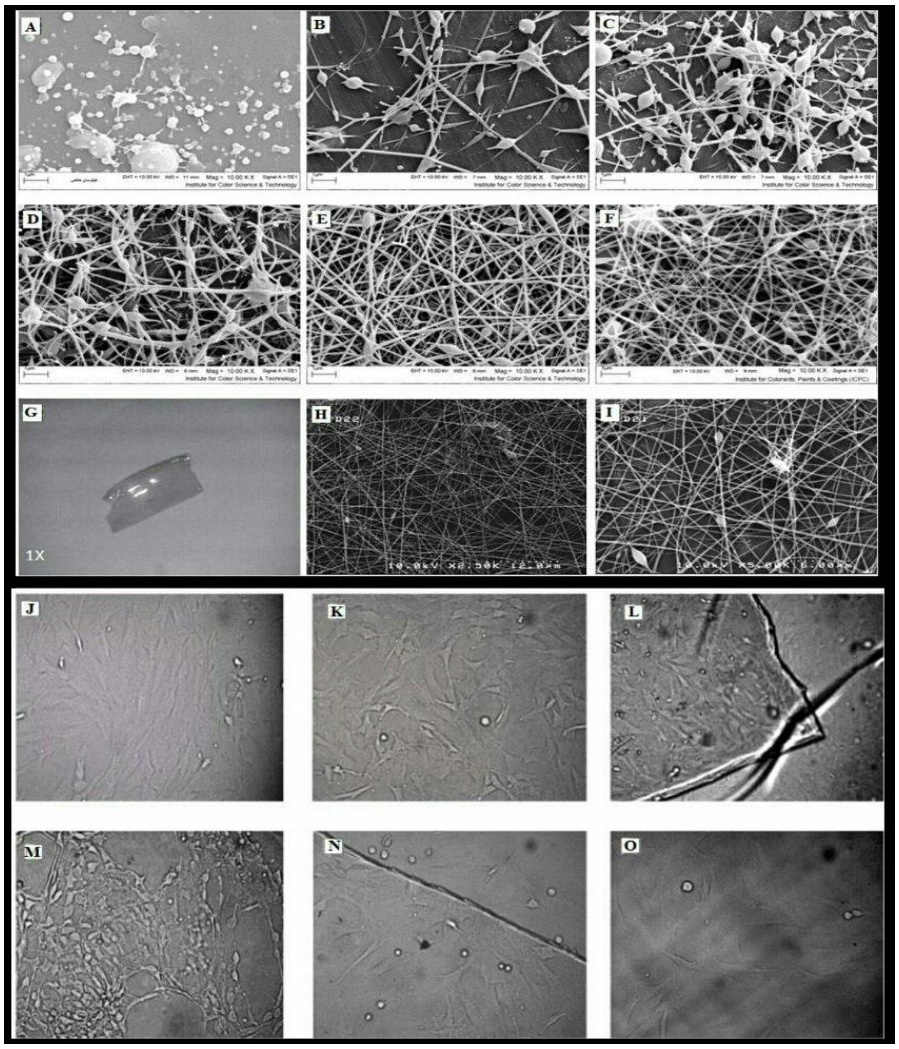

Fig 2. SEM images of CS/PEO electrospun nanofibers at different concentrations: (A) 100/0; (B) 90/10; (C) 80/20; SEM images of CS/PEO electrospun nanofibers containing 0.3% Triton X-100 at different concentrations: (D) 100/0; (E) 90/10; (F) 80/20; (G) CTF at 1X magnification; CS/PEO electrospun nanofibers on CTF at different concentrations: (H) 90/10 and (I) 80/20; Photomicrographs of BMSCs were seeded on CS film/nanofibers membranes of CS/PEO (90/10) at the sixth day of culture at 200X magnification: (J) Control, (K) CTF, (L) Nanofibers 90/10 on CTF, (M) Nanofibers 80/20 on CTF, (N) Nanofibers 90/10 without CTF and (O) Nanofibers 80/20 without CTF

PEO. This peak is gradually shifted to higher wave numbers by increasing the PEO content in the nanofiber. Therefore, strong interactions between CS and PEO may prevail the formation of these hydrogen bonds.

Electrospinning

Fig 2 (A-C) illustrates the maximum CS/PEO ratio for making a spinnable solution, above which the spun product exhibited a non-uniform structure. A structure of short fibers embedded with a considerable amount of beads was seen. As shown in Fig 2 (D- F), a small amount of Triton X-100 and DMSO improved the spinnability of the polymer solutions CS/PEO 90/10 and 80/20 and prepared the nanofibers 220±10 nm and 240±10 nm, respectively.

The fibers were improved compared to the previous ones. However, the 100/0 ratio of CS/PEO (Fig 2D) was not able to produce the typical nanofiber. Fig 2G shows CTF at 1X magnification. Fig 2 (H and I) displays the nanofibers 90/10 and 80/20 on CTF, respectively.

We assessed the number of cells at the sixth

day after cell culturing on the film and nanofiber. The confluency rates of cells on the sixth day in control (Fig 2J), CTF (Fig 2K), nanofiber 90/10 on CTF (Fig 2L), and nanofiber 80/20 on CTF (Fig 2M) were filled, while those of nanofiber 90/10 without CTF (Fig 2N) and nanofiber 80/20 without CTF (Fig 2O) were about 90±3 percent.

The shape and position of the cell in the CTF (without nanofiber) containing cells seemed slightly wider and more sticky than nanofiber 90/10 on CTF, nanofiber 80/20 on CTF, nanofiber 90/10, and nanofiber 80/20.

Fig 3 shows the comparison of the percentage of cell proliferation on the nanofiber 90/10, nanofiber 80/20, nanofiber 90/10 on CTF, nanofiber 80/20 on CTF, CTF, and control groups on different days.

Cell proliferation in CTF group was like that of the control group, and there were significant differences between the second day and the fourth and sixth days.

However, there were no significant differences between the fourth and sixth days. No significant differences were found in the fourth and sixth days among the nanofiber 90/10, nanofiber 80/20, CTF, and control groups.

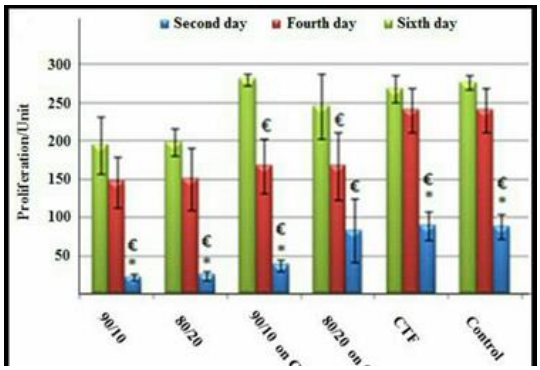

Fig 3. Comparison of cell proliferation diagram in different groups on the second, fourth and sixth days

Cell viability

In this method, stain penetrated into the dead cells and was seen in blue color, while the living cells were colorless.

After the second, fourth, and sixth days from the third passage of the BMSCs on the nanofiber and CTF, the viability of the cells was 90±3% (Table 1).

Table 1. Viability of BMSCs in different groups

Samples	Viability of BMSCs		
	Second day	Fourth day	Sixth day
Control	93.2±1.0	95.2±1.5	97.4±2.4
CTF	90.8±1.7	93.6±1.4	94.6±3.0
Nanofiber 90/10 on CTF	90.2±1.1	88.0±1.5	89.0±1.4
Nanofiber 80/20 on CTF	89.8±1.0	91.4±3.5	90.0±2.6
Nanofiber 90/10	91.4±1.8	91.8±2.1	92.0±1.9
Nanofiber 80/20	92.2±1.9	93.0±2.3	92.8±2.0

Immunocytochemistry

The cellular phenotype was characterized by immunocytochemistry for fibronectin and CD44 (Fig 4).

The percentages of immunoreactive cells were 95.48±0.24% and 97.16±0.82%, respectively. Also, none of or very few cells expressed CD45.

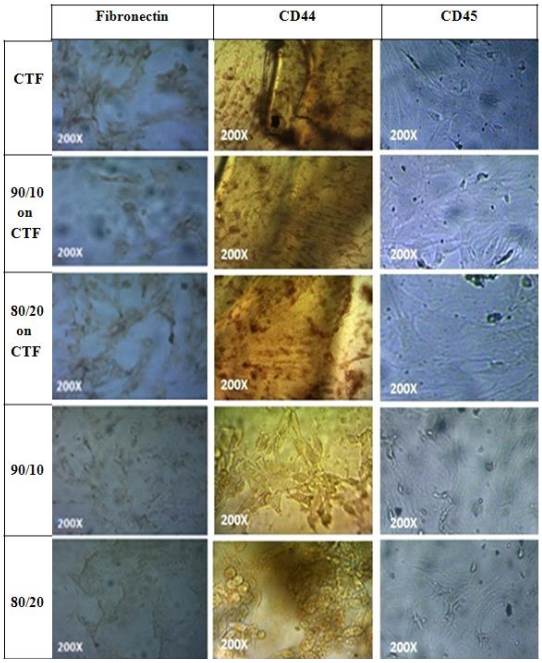

Fig 4. Characterization of the undifferentiated bone marrow stromal cells using immunocytochemistry at 200X magnification. The differentiation markers used in the study included fibronectin (a marker for BMSCs) and CD44 (markers of mesenchymal stem cells). They were incubated with anti-fibronectin, anti-CD44 (primary antibodies), followed by the secondary staining with DAB reagent

Scanning electron microscopy (SEM)

Fig 5A shows the SEM images of the BMSCs grown on the CS thin film without nanofibers and Fig 5 (B and C) show the SEM images of the

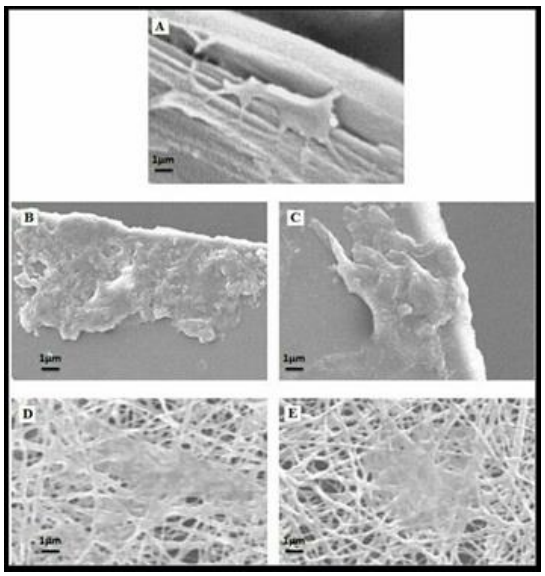

Fig 5. SEM images of bone marrow stromal cells attachment on day 6 of incubation: (A) Chitosan thin film without nanofibers; (B, C) Chitosan thin film/nanofiber scaffolds and (D, E) CS/PEO nanofiber scaffolds

BMSCs were grown on CS thin film with nanofibers after 6 days in the culture medium. The cells were attached to the surfaces and exhibited some long microvilli on their surfaces. The nanofibers and films were merged together after 6 days. Figure 5 (D and E) illustrates the SEM images of the BMSCs grown on CS/PEO nanofiber scaffolds after 6 days in the culture medium.

The cells adhered well and exhibited the characteristic round shape of BMSCs, indicating that the nanofibers maintained the phonotype of BMSCs. These results indicate that the CS/PEO nanofiber scaffold supports the cell attachment and proliferation; hence, these scaffolds are useful for tissue engineering applications.

DISCUSSION

Different behavior of CS and PEO in electrospinning was attributed to their intrinsically different nature in solution. The success of CS/PEO-assisted electrospinning is believed to be the consequence of the strong hydrogen bonds formed between the ether groups in PEO and the hydroxyl and amino groups in CS, as shown by FTIR. Electrospinning at 24˚C also helped to stabilize the jet and improved the spinability of CS solutions. It was found that increasing the CS content in the blend solutions led to a significant reduction in nanofiber diameters 240±10 nm and 220±10 nm for CS/PEO blends 80/20 and 90/10, respectively at 24˚C [16, 17].

The SEM images further confirmed that CS/PEO nanofibers and CS films promoted the adhesion of BMSCs and maintained the characteristic cell morphology. Thus, cell phenotype may serve as a potential candidate for bone tissue engineering. Kong et al. reported that the pre-osteoblast cells cultured on the apatite-coated scaffolds showed a different behavior. The cells presented better proliferation on the apatite-coated CS/Nano-hydroxyapatite composite scaffolds than on CS/apatite-coated scaffolds [18].

Bhattarai et al. reported that the CS/PEO nanofiber scaffolds promoted the attachment of human osteoblasts and chondrocytes and maintained the characteristic cell morphology and viability throughout the study period. This nanofiber matrix is of particular interest in tissue engineering for controlled drug release and tissue remodeling [14].

In this study, the SEM images of CS/PEO nanofibers showed that these fibers with diameters ranging from 220 nm to 240 nm provided suitable bonding capabilities for cultivation and cell growth, including BMSCs, in vitro. This finding is somewhat in agreement with the results of Kaka et al. as they could culture BMSCs on the nanofiber scaffold poly L-lactic-co-glycolic acid (PLGA) to improve transected sciatic nerve regeneration [12].

Kazeminejad et al. showed the possibility of human bone marrow mesenchymal stem cell proliferation and differentiation into hepatocytes on the nanofiber scaffolds formed by polycaprolactone (PCL), collagen, and polyethersulfone (PES) [19].

In the present study to distinguish the purity of stromatic cells due to the presence of glycoprotein fibronectin in mesenchymal-originated cells, BMSCs were stained against this glycoprotein using immunocytochemistry method. High expression of fibronectin in the cells confirmed they are stem cells [20].

To confirm the purity of BMSCs, CD44 antibodies were used, and the results indicated a high percentage of positive cells for the fibronectin antibody. This result has also been observed by others regarding mesenchymal stem cells [21]. Through application of anti-fibronectin antibody and mRNA expression of Oct-4 gene, Lamoury et al. cultured the BMSCs of animals and humans in two separate media and verified that they were

stem cells [22].

The strength of this study was to produce an appropriate coverage in wound healing and tissue regeneration.

We did not perform tests on laboratory animals therefore we are cannot discuss their effectiveness and possible side effects in vivo. This is considered as the limitation of this study.

CONCLUSION

This study summarized the preparation and tissue engineering applications of CS-based nanofibers. Further studies are required to explore the clinical applications and commercialization of the CS-based nanofibers.

On the other hand, few BMSCs deaths were observed when they were co-cultured with CS Nanofiber/Film Scaffolds. The viability, morphology, and undifferentiated properties of BMSCs were maintained unaffected in this study. Therefore, biodegradable CS/PEO nanofibers and thin film scaffolds are suitable models in tissue engineering, wound dressing and cell therapy.

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ETHICS STATEMENT

The ethics committee of the Shahid beheshti University of Medical Sciences, Tehran, Iran, approved this study (IR.SBMU.MSP.REC.1397.437).

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