Original Article
Evaluation of osteoconductive effect of polycaprolactone (PCL) scaffold treated with *fibronectin* on adipose-derived mesenchymal stem cells (AD-MSCs)

Reyhaneh Saadat Rezaee Asl[1], Fatemeh Rahimzadeh-Bajgiran[1], Ehsan Saburi[2,3]

[1]Department of Biology, Mashhad Branch, Islamic Azad University, Mashhad, Iran; [2]Medical Genetics Research Center, Mashhad University of Medical Sciences, Mashhad, Iran; [3]Department of Medical Genetics and Molecular Medicine, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

Abstract: Background: Replacing damaged organs or tissues and repairing damage by tissue engineering are attracting great interest today. A potentially effective method for bone remodeling involves combining nanofiber scaffolds with extracellular matrix (ECM), and growth factors. Today, electrospun PCL-based scaffolds are widely used for tissue engineering applications. Methods: In this study, we used an electrospun polycaprolactone (PCL) scaffold coated with fibronectin (Fn), a ubiquitous ECM glycoprotein, to investigate the induction potential of this scaffold in osteogenesis with adipose-derived mesenchymal stem cells (AD-MSCs). Results: Scanning electron microscopy (SEM) analysis showed that fibronectin, by binding to the membrane receptors of mesenchymal stem cells (MSCs), leads to their attachment and proliferation on the PCL scaffold and provides a suitable environment for osteogenesis. In addition, biochemical tests showed that fibronectin leads to increased calcium deposition. The results also showed that alkaline phosphatase activity was significantly higher in the PCL scaffold coated with fibronectin than in the control groups (PCL scaffold group and tissue culture polystyrene (TCPS) group) ($P<0.05$). Also, the analysis of quantitative reverse transcription PCR (qRT-PCR) data showed that the relative expression of bone marker genes such as osteonectin (ON), osteocalcin (OC), RUNX family transcription factor 2 (RUNX2), and collagen type I alpha 1 (COL1) was much higher in the cells seeded on the PCL/Fn scaffold than in the other groups ($P<0.05$). Conclusions: The results show that fibronectin has an increasing effect in accelerating bone formation and promising potential for use in bone tissue engineering.

Keywords: Bone tissue engineering, mesenchymal stem cells, polycaprolactone, fibronectin

Introduction

Bone is one of the hardest types of connective tissue [1]. The particular composition and different structures of bone determine its important functions in humans, which mainly include protection, support, movement, storage, and production of blood cells [2]. However, sometimes bone damage (fibrous dysplasia, osteosarcoma, multiple myeloma, and chondrosarcoma) is so widespread or the damage is so severe that rebuild is not possible on its own or even with medication and surgery [3]. Most bone grafts today are used to repair bone defects [4]. Bone grafts are limited due to problems such as finding suitable connective tissue, the need for re-surgery, and immune reactions. In these cases, tissue engineering can be used to repair the injury [5]. Replacement of damaged tissue-related organs and repair of damage by tissue engineering have received a lot of interest [6]. Also, Stem cell-based treatment is proposed as a new tactic in tissue engineering [7]. Adipose tissue is one of the most available and rich sources of mesenchymal stem cells (MSC) [8]. Studies have shown that in the same amount of adipose tissue and bone marrow, the number of MSCs in adipose tissue is 500 times higher [9]. This means that MSCs from adipose tissue are among the most suitable cells for regenerative medicine [10]. Application and source of mesenchymal stem cells derived from fat in wound and scar treatmet [11] and regenerative medicine [12]. In this study, the application of adipose-derived mesenchymal stem cells (AD-MSC) in bone tissue

engineering is that after separating the stem cells from the adipose tissue of patients such as surgery and suction, its effectiveness can be checked on the Polycaprolactone (PCL) scaffold treated with Fibronectin (Fn).

In addition to suitable cells, other important components for tissue engineering include scaffolds, which have a wide variety and are engineered with the ability to mimic natural tissue structures for tissue regeneration [13, 14]. One of the most commonly used biopolymers is PCL [15], which has properties such as biocompatibility, biodegradability, no cytotoxicity, easy preparation, suitable physical and mechanical properties, and good stability, and is used for the construction of engineering scaffolds [16]. To create an environment similar in vivo, PCL can be modified by combining it with materials such as synthetic or natural polymers to improve its properties [17]. Electrospinning is among the technologies that produce these structures similar to the natural extracellular matrix [18]. Extracellular matrix proteins commonly used in electrospun PCL-based scaffolds include Fn [19]. Fn has functions such as cell adhesion, regulation of cell activity, and strong binding of cells to polymers [20]. Polymeric nanofibers such as PCL increase the absorption of certain proteins such as Fn, which leads to better cell attachment to the nanofibers and cell expansion during tissue engineering [21]. Studies have been conducted in the field of modifying the properties of PCL polymer with different materials [22]. For example, Gupta et al. demonstrated improved adhesion and proliferation of Schwann cells with aligned PCL/gelatin-based electrospun fibers [23]. Also, by modifying PCL with chitosan/lidocaine hydrochloride, Li X et al. showed that this scaffold leads to increased hydrophilicity, cell compatibility, and sustained drug release [24]. Today, electrospun PCL-based scaffolds are used in tissue engineering applications as support scaffolds for bone, skin, and cartilage [25]. Thus, in the present study, we investigated the osteoconductive effect of the PCL scaffold treated with Fn on AD-MSC.

Materials and methods

Preparation of scaffold

The PCL scaffold was fabricated using electrospinning technology, as described previously [26]. 680,000 g/mol PCL powder was added to 10 ml solution containing 7.5 mL chloroform and 2.5 mL N-dimethylformamide (DMF). The polymer was placed in a 5 ml nozzle at a distance of 15 to 18 cm from the collector of the device and was thrown in the electric field with a voltage of 21 kV onto the plate and a specific target. The polymer solution was evaporated in the path of the electric field and porous fibers were obtained. The fabricated scaffold was sterilized with ethanol 70% and UV and fibronectin were applied to the PCL scaffolds per 1 cm^2 dimensions.

Scaffold hydrophobicity and morphological properties

The hydrophobicity properties of the scaffold were tested at room temperature using a contact angle conveyor (Krüss, Hamburg, Germany). In addition, the morphology of the scaffold was observed with Scanning electron microscopy (SEM) (S-4500; Hitachi, Tokyo, Japan) (**Figure 1**).

Cell culture

As described previously, MSCs were obtained and isolated from adipose tissue (ACECR, Mashhad, Iran) [27]. Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) low glucose (Gibco, US) and 15% (v/v) fetal bovine serum (FBS, Gibco, US) and 1% (v/v) Penicillin and streptomycin (Pen/Strep) (complete medium) and incubated at 37°C and 5% CO_2. Subsequently, the third passage of cells was seeded in 48-well plates (15×10^3 cells per well) to assess the ability of AD-MSCs to differentiate into the osteocyte and adipocyte lineages.

The ability of AD-MSCs differentiation

Cells were divided into two groups and cultured for 21 days in osteogenic differentiation medium (10 mM glycerol phosphate, 50 ug/ml ascorbic acid, 10 nM dexamethasone, and 10 nM vitamin D3 (all from Sigma)), and 10% FBS and adipose differentiation medium (100 nM dexamethasone and 50 mg/L indomethacin, and 10% FBS). The medium of the cells was changed every other day and the cells were harvested 21 days after cell seeding for alizarin red and oil red staining.
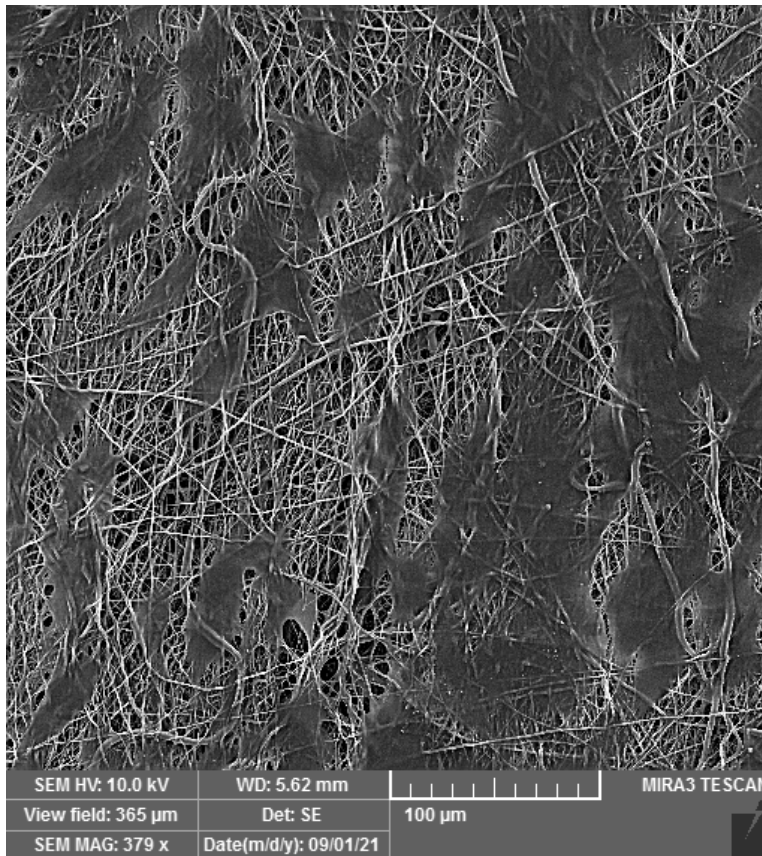
Figure 1. The AD-MSC cultured on PCL coated with fibronectin (magnification 379X).

The test was performed on 3 studied groups. 1-The group of cells seeded on the PCL scaffold, 2-The group of cells seeded on the PCL/Fn scaffold, 3-The cells of the TCPS (Tissue Culture Polystyrene) group. Cell viability was evaluated in 3 time periods of 1, 3, and 5 days using the resazurin assay (7-hydro-xy-10-oxido-phenoxazin-10-ium-3-one) as follows. Cells were plated into 96-well plates (12×10^3 cells per well). At the desired time intervals, 200 µl complete medium and 50 µl resazurin were added to each well. After incubation in a CO_2 incubator for 2 hours, optical density (OD) was read at 600/570 nm using Epoch Plate Reader (BioTek, United States).

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured in the 3 mentioned groups according to the instructions of the Pars Azmoun ALP kit (Pars Azmoun, Iran). On days 7, 14, and 21, cells were lysed with radioimmunoprecipitation (RIPA) lysis buffer, and after centrifugation at 15,000 rpm for 15 minutes at 4 degrees, reagents R1 and R2 were added, and optical density (OD) was read at 405 nm. The ALP activity was expressed as a ratio to total protein for each sample.

Calcium content

To measure the calcium content, the cells of the studied groups were lysed with HCL (0.6 N), and based on the kit used (Pars Azmoun, Iran). Then OD was read at 570 nm and the results were interpreted according to the protocol of Arab et al. [28].

Evaluation of osteogenic differentiation of AD-MSCs

To measure the expression of bone marker genes, including ON, OC, RUNX2 and COL1, mRNA extraction was performed from AD-

Oil red staining

Cells were washed three times with Phosphate buffered saline (PBS) and fixed with formaldehyde (10%) and incubated for 10 minutes. Then, isopropanol (60%) was added and incubated for 5 minutes. Finally, a drop of oil red dye (Sigma) was added and after 10 minutes, the cells were washed with PBS and observed under an inverted microscope.

Alizarin red staining

The cells were fixed in formaldehyde (4%) at room temperature for 1 hour. The dye alizarin red (Sigma) was added to the cells. After 10 minutes, the cells were washed with PBS and observed with an inverted microscope.

Cell viability assay

After confirming the differentiation ability of the AD-MSCs, the cells were seeded on the fabricated scaffolds to evaluate the cell viability.

Table 1. Primers for real-time PCR quantification of osteogenic genes

Gene	Primer Sequences (5'-3')	Product Size (bp)
Beta 2 Microglobulin-Forward	TGGAAAGAAGATACCAAATATCGA	201
Beta 2 Microglobulin-Reverse	GATGATTCAGAGCTCCATAGAGCT	
Collagen I-Forward	TGGAGCAAGAGGCGAGAG	121
Collagen I-Reverse	CACCAGCATCACCCTTAGC	
Runx2-Forward	GCCTTCAAGGTGGTAGCCC	66
Runx2-Reverse	CGTTACCCGCCATGACAGTA	
Osteonectin-Forward	AGGTATCTGTGGGAGCTAATC	224
Osteonectin-Reverse	ATTGCTGCACACCTTCTC	
Osteocalcin-Forward	GCAAAGGTGCAGCCTTTGTG	80
Osteocalcin-Reverse	GGCTCCCAGCCATTGATACAG	

MSCs using an RNA extraction kit (Parstous, Iran) and cDNA was synthesized using cDNA kit (Parstous, Iran). Changes in gene expression were quantified by real-time PCR according to the instructions of the kit (AMPIQON, Denmark). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method against the reference gene Beta 2 Microglobulin (B_2m). Primers for bone marker genes are listed in **Table 1**.

Statistical analysis

The result of the tests was checked with one-way ANOVA test. GraphPad Prism 9 software (GraphPad Software, Inc., La Jolla, California) was used to analyze the data, and *P* values ≤0.05 were considered significant. Data were expressed as mean ± standard deviation (SD).

Results

Scaffold characterization

The microstructure of the PCL coated with Fn was evaluated by SEM. The results showed that Fn increased the hydrophilic and biocompatible properties of the electrospun PCL scaffold (**Figure 1**). SEM images showed that electrospun PCL fibers after treatment with Fn had not induced any significant changes in scaffold characteristics as pore size, bead free and smooth surfaces.

AD-MSC differentiation potential

Oil-red and Alizarin-red staining: The ability of AD-MSCs to differentiate into two cell lineages, osteogenic and adipogenic after 21 days was measured by alizarin red and oil red was staining, respectively. The results showed that the fat vacuoles in the cytoplasm of cells differentiated into adipocytes were stained red, indicating adipogenesis (**Figure 2B**). Also, observation of cells differentiated into osteocytes under the inverted microscope showed that the reaction of calcium deposited in the cytoplasm of the cells with alizarin leads to the formation of alizarin red S-calcium complex, which was observed in the form of red masses (**Figure 2A**).

Cell viability

Resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) is an indicator for cell viability assays by identifying mitochondrial activity and is suitable for long-term cell cultures. The same results in terms of cell viability were observed between the 3 groups PCL-scaffold, PCL/Fn-scaffold, and TCPS on days 1 and 3, whereas a significant difference in TCPS was observed on day 5 (P<0.0001). **Figure 3** which is probably due to cell proliferation and growth after 5 days. Thus, the results showed that both the PCL scaffold and the fibronectin-coated on it had no toxicity to the seeded cells (**Figure 3**).

Calcium content and alkaline phosphatase activity

The results of cellular calcium content and ALP activity as indicators of osteoblastic differentiation are shown in **Figures 4** and **5** respectively. Calcium content of the PCL scaffold was significantly higher than that of the PCL/Fn scaffolds and TCPS 21 days after differentiation. Calcium deposition of seeded cells on the PCL/Fn scaffold also showed a significant increase on day 21 compared to TCPS (**Figure**
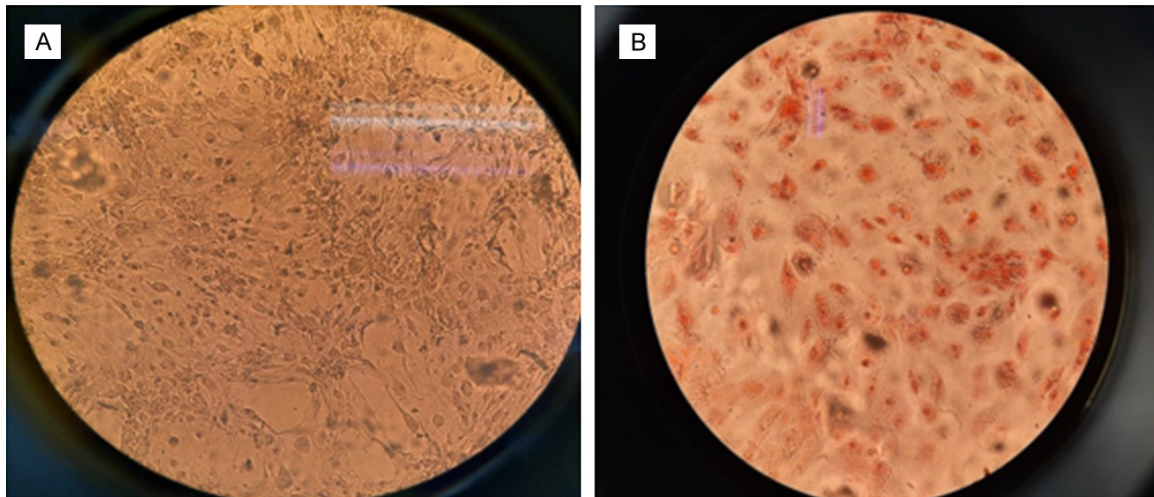
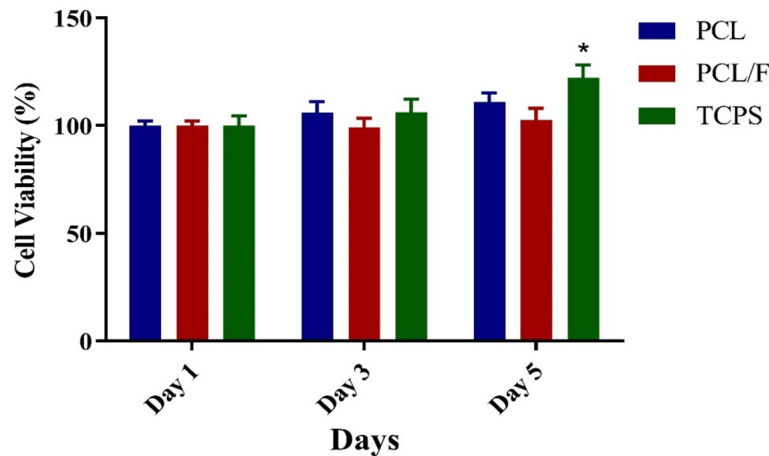**Figure 2.** Alizarin-red staining (A). Oil-red staining (B). 400X magnification.

Figure 3. Cell viability. Both Polycaprolactone and Polycaprolactone/fibronectin scaffolds showed no lethal effect on mesenchymal stem cells (*P<0.0001).

the PCL/Fn scaffold compared with the other groups in all 3-time points (P<0.001). The expression of all mentioned genes also showed a significant increase in all 3-time intervals compared with TCPS (P<0.001).

Discussion

Regenerative medicine with stem cell-based therapy and tissue engineering has created a new solution for healing irreparable injuries such as bone injuries. Due to a variety of bone injuries (fractures, osteoporosis, osteomalacia, etc.), the regeneration of bone tissue is nowadays paid great attention in research. The use of scaffolds in various studies is increasing due to their ability to mimic natural tissue structures. The combination of scaffolds with ECM, natural and synthetic polymers, and growth factors has shown promising results in bone remodeling in the context of bone tissue engineering [29]. Electrospun nanofiber scaffolds form a suitable substrate for cell attachment, proliferation, and differentiation due to several properties such as biocompatibility, biodegradability, no cytotoxicity, and easy fabrication.

4) (P<0.0001). Examination of alkaline phosphatase activity on days 14 and 21 after cells were cultured on the scaffolds and TCPS showed that the ALP activity of PCL/Fn scaffold was significantly increased compared with the other two groups, suggesting that the fibronectin-modified PCL scaffold plays a positive role in the process of osteogenesis (**Figure 5**).

Expression of osteogenic genes

Relative expression of osteogenic markers was assessed at differentiation time points (in days 7, 14, and 21) as shown in **Figure 6**. The results showed that the expression of COL1, OC, ON, and RUNX2 genes were significantly higher in

Among the cells cultured on electrospun scaffolds in tissue engineering include mesenchy-
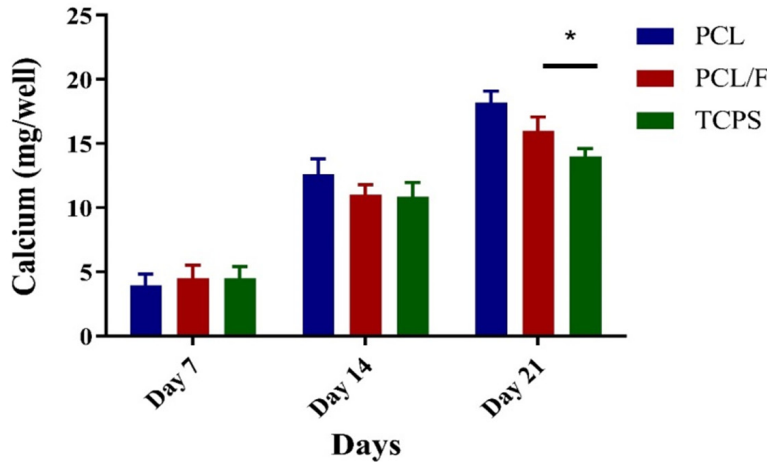
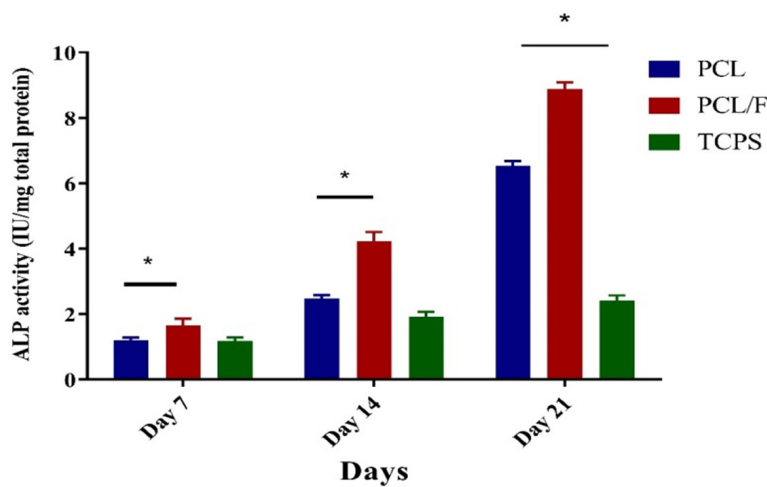Figure 4. Calcium content in 3 time periods and 3 groups (*P<0.0001).

Figure 5. Alkaline phosphatase activity (*P<0.0001).

mal stem cells. In a study, Wang et al., showed that the use of mesenchymal cells on polymeric nanofibers such as PCL can improve bone regeneration [4]. The use of 3D scaffolds, including PCL, for bone tissue engineering is becoming more extensive by the day. Rumiński et al., confirmed that seeding MSCs on PCL scaffold leads to increased osteogenic differentiation of MSCs [30]. In the present study, we used PCL scaffold to provide a substrate for the differentiation of AD-MSCs into osteocytes.

The role of ECM proteins in the healing of bone fractures has been demonstrated in various studies [31-33]. One of the most important of these proteins is FN, which acts as a regulator and plays a role in various stages of fracture healing [34]. And it provides a platform for the function of other ECM components in fractures, and also helps in cell absorption, proliferation, and differentiation [35]. Also, the results of our study showed that Fn causes AD-MSCs trapped in the PCL scaffold and leads to the induction of osteogenesis by regulating cellular behavior. In confirmation of the results of our study, Klavert et al., after examining FN-based biomaterials showed that fibronectin probably has an important function in different stages of bone fracture healing [36]. Likewise et al., showed that fibronectin can also regulate osteoclast activity [37].

In the present study, calcium deposition, ALP activity, and expression of bone marker genes showed a significant increase in cells seeded on the PCL/Fn scaffold, confirming the osteogenic induction ability of the PCL scaffold coated with Fn. Wang et al., showed that fibronectin and cadherin-11 (CDH) play important roles in osteogenesis and cell adhesion. They confirmed that the proliferation and differentiation of human bone marrow mesenchymal stem cells (hMSCs) increased on the PLGA/collagen scaffold, and the expression of bone marker genes (ALP, RUNX2, OC) showed a significant increase [38]. In addition, Kang et al., demonstrated that seeding umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) on Fn-treated PCL scaffold resulted in improved cardiac function [39]. Mohamadyar-Toupkanlou et al., have also shown that PCL/nHA nanofiber scaffold coated with Fn can increase the expression of bone marker genes and lead to greater calcium deposition and increased ALP activity [40]. The results of our study and the studies performed show that the PCL scaffold coated with fibronectin can be considered a reliable candidate for bone tissue engineering and the improvement of bone
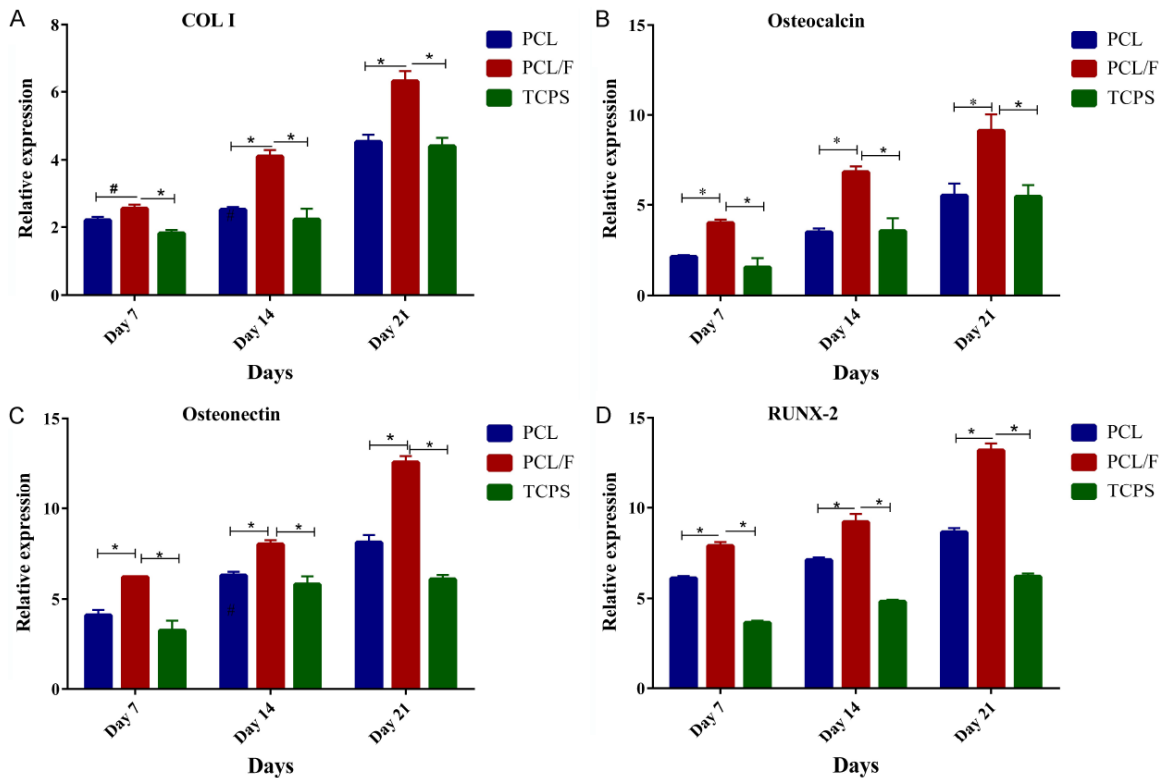
Figure 6. Relative expression of collagen-I (A), osteocalcin (B), osteonectin (C), Runx2 (D) on PCL, PCL/FN, and TCPS at 7, 14 and 21 days after cell seeding (*P<0.001, #P<0.05).

defects. However, further studies are needed on the mode of action of fibronectin and the molecular pathways by which it exerts its effect on osteogenesis. In research using silk with glycidyl methacrylate to produce artificial blood vessels is currently under investigation. Finally, silk vessels have shown a high degree of openness and a degree of satisfaction with the coverage of endothelial cells [41]. In that study, a promising candidate peptide for improving the therapeutic potential of hWJ-MSCs is the peptide derived from FGF-2 FP2, and it was especially expressed in bone and cartilage regeneration [42]. Gatto et al., in a study concluded that HA has a positive effect on hMSC adhesion, while on the contrary, it has a negative effect on scaffold density under pressure [43]. Gupta et al., in a research emphasizes the multifaceted effect of fucoidans on osteoprogenitor cells and highlights the delicate balance between potential therapeutic benefits and challenges in using fucoidans for post-surgical treatments in patients with osteosarcoma [44]. Here, ALP activity was significantly higher in osteogenic environment compared to proliferation, which

indicates hMSC differentiation towards osteoblasts [45].

Conclusion

The results of this study have shown that PCL modified with Fn has excellent osteogenic induction potential by increasing the expression of bone marker genes, the activity of ALP, and calcium deposition. Therefore, it has promising potential for use in bone tissue engineering. It seems that considering the important and fundamental role of fibronectin in the strength and formation of the ECM and considering the vital role of the strength of the ECM in the formation, density and efficiency of bone, it seems that fibronectin can play an effective role in the differentiation of stem cells into osteogenic cells.

Limitation

However, it is important to note that this study focused on in vitro evaluations and experiments. Although it provided valuable insights into the osteoconductive effect of the Fn-

treated scaffold on adipose-derived mesenchymal stem cells, further studies in animal models and humans are needed to validate these findings.

Acknowledgements

Disclosure of conflict of interest

None.

Abbreviations

AD-MSCs, adipose-derived mesenchymal stem cells; COL1, collagen type I alpha 1; ECM, extracellular matrix; Fn, fibronectin; FBS, Fetal Bovine Serum; hMSCs, human bone marrow mesenchymal stem cells; MSCs, Mesenchymal Stem Cells; OD, Optical density; ON, Osteonectin; OC, Osteocalcin; PCL, Polycaprolactone; Pen/Strep, Penicillin and Streptomycin; PBS, Phosphate Buffered Saline; SEM, Scanning electron microscopy; TCPS, tissue culture polystyrene.

Address correspondence to: Ehsan Saburi, Department of Medical Genetics and Molecular Medicine, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran. Tel: +98-9151048897; E-mail: ehsansaburi@yahoo.com; Sabourie@mums.ac.ir

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