Comparison of proliferation and osteoblast differentiation of marrow-derived mesenchymal stem cells on nano- and micro-hydroxyapatite contained composite scaffolds

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Abstract

Bones constructed by tissue engineering are being considered as valuable materials to be used for regeneration of large defects in natural bone. In an attempt to prepare a new bone construct, in this study, proliferation and bone differentiation of marrow-derived mesenchymal stem cells (MSCs) on our recently developed composite scaffolds of nano-, micro-hydroxyapatite/ poly(*l*-lactic acid) were compared with pure poly(*l*-lactic acid) scaffolds. For this purpose, some passaged-3 rat MSCs were three-dimensionally cultivated on the scaffold surfaces and their morphology was observed with scanning electron microscopy. Cell proliferations on different scaffolds were examined by MTT assays. Osteogenic cultures were established with the scaffolds loaded with MSCs for 21 days at the end of which culture mineralization; the cell alkaline phosphatase (ALP) Level and the relative expression of selected bone specific genes were quantified and compared to each other. Our results indicated that the cells having been adhered and assumed spherical morphology were able to proliferate in all studied scaffolds. The microenvironment provided by nano-scaffolds appeared much better medium than those of micro-scaffolds and pure PLLA ($P < 0.05$). The osteogenesis assays indicated to the superiority of nano-scaffolds in supporting MSCs undergoing bone differentiation, which was reflected in high cellular ALP levels, increased bone-related gene expression and enhanced culture mineralization. Collectively, the bone construct prepared with nano-hydroxyapatite/ poly (*l*-lactic acid) scaffold and proliferated MSCs would be suitable candidate for use in bone regenerative medicine.

Keywords**:** nano- and micro-hydroxyapatite; poly (*l*-lactic acid); Mesenchymal stem cell; Bone differentiation; Cell proliferation; Scaffold

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INTRODUCTION

Large bone defects represent major clinical problems in the practice of reconstructive orthopedic and craniofacial surgery. In such situation, bone grafts and/or bone substitutes are preferred (Logeart-Avramoglou *et al*., 2005). Autografts are biocompatible, but their use is limited due to the lack of tissue supply, and also because the pain and morbidity often develop at the donor site (Ito *et al*., 2005; Wiedmann-Al-Ahmad *et al*., 2005). Allogenic graft could be regarded as an alternative option for repair of large bone defects, but it presents the risk of disease transmission, and may trigger host immune response (Weinand *et al*., 2006). The use of metal implant has two fundamental limitations: the need for secondary operation to remove implant and the possible releasing of metal ions and toxic compounds by implant itself (Chen *et al*., 2003; Savarino *et al*., 2008). Therefore, elaboration of appropriate bone replacement materials would be of consid-

erable importance. Currently bone construct created by tissue engineering principles using an appropriate scaffold and cellular materials is considered as an ideal bone substitute (Lu *et al*., 2010; Mikos *et al*., 2006).

Until now, osteoblastic as well as mesenchymal stem cells have gained fairly extensive attention as cellular candidates for bone tissue engineering (Gordeladze *et al*., 2009; Wiesmann *et al*., 2003). Osteoblastic cells are differentiated bone cells with limited proliferation capacity, while MSCs are adult stem cells representing extensive replication potential. MSCs possess two important properties of extensive self-renewal ability and multilineage differentiation potential that make them more suitable for tissue engineering application. Moreover these cells can easily be isolated from bone marrow aspiration which conventionally involves the insertion of needle into iliac crest thus leaving relatively less morbidity than the either bone or periosteum harvest (Heng *et al*., 2004; Eslaminejad *et al*., 2006 and 2007).

There are several biocompatible compounds that can be used in bone tissue engineering as scaffolds. These include polymers, ceramics and composite materials. In the earlier attempts, ceramics such as coralline and synthetic hydroxyapatites and β-tricalcium phosphate were examined for bone tissue engineering. Moreover, polymer scaffolds such as poly*(l*-lactic acid) (PLLA), poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and polylactic-*co*-glycolic (PLGA) have also gained considerable attentions as scaffolds for bone engineering (Eglin *et al*., 2008; Cerroni *et al*., 2002; Zhang *et al*., 2009; Hu *et al*., 2003; Newman *et al*., 2004; Rezwana *et al*., 2006).

Hydroxyapatite (HAP) exhibits composition and structure that is very similar to bone mineralized matrix. This compound possesses bioactivity, biodegradability and high osteoconductivity. Nevertheless HAP is a fragile compound with very slow degradation rate *in vivo* and low osteoinductive potential (Cerroni *et al*., 2002). PLLA is a biodegradable synthetic polymer being used in orthopedic surgery. Moreover, PLLA possesses suitable and predictable degradation rate. In spite of these valuable advantages there are limitations associated with their surface properties for cells attachment and proliferation. In addition, PLLA degradation *in vivo* may release acidic compounds which result in inflammatory response (Ishii *et al*., 2009).

Composite scaffolds of HAP and PLLA have been shown to possess more biocompatibility than each individual scaffold. According to a study, about half of cultivated cells undergo apoptosis when being seeded on pure PLLA surfaces at early days of culture (Ignjatovi *et al*., 2001; Najman *et al*., 2004). Based on several reports, osteoblastic cell lines adhere on HAP/PLLA scaffolds better than pure PLLA scaffold (Sui *et al*., 2007; Deng *et al*., 2007). In the previous study, we developed composite scaffolds consisting of nano-hydroxyapatite/poly (*l*-lactic acid) (nHAP/PLLA) and micro-hydroxyapatite/poly (*l*-lactic acid) (mHAP/PLLA) (Nejati *et al*., 2009). Our investigation showed that nHAP/PLLA had better mechanical properties compared to mHAP/PLLA composite. In the present study, the scaffolds are compared in terms of their microenvironmental condition to support MSCs proliferation and osteogenic differentiation.

MATERIALS AND METHODS

Scaffolds: The scaffolds were made according to our previous method (Nejati *et al*., 2009). Briefly, the nHAP was prepared by wet chemical method using Ca $(NO_3)_2.4H_2O$ (Merck, Germany) and $(NH_4)_2HPO_4$ (Merck, Germany) as Ca and P precursors, respectively. A 0.03 M aqueous solution of $(NH_4)_2HPO_4$ was added dropwise to a 0.05 M aqueous solution of Ca $(NO_3)_2.4H_2O$ under stirring to form nHAP particulates. In all experiments the pH solution was adjusted to 11 by diluted NaOH solution (Merck, Germany) and the temperature was maintained at 70±5°C. The precipitated HAP particles were aged for 24 h at room temperature. The resulting product was centrifuged at the rotation speed of 9000 rpm for 30 min using a Sigma-12151 rotor.

nHAP/PLLA Composites were made by the following procedure: synthesized nHAP powder was dispersed in a 10 ml 1,4-dioxane (Merck, Germany) by sonication for 2 min at 130 W (Bandelin Sonoplus HD2200, Berlin, Germany). Then 0.5 g PLLA (IMI, Canada) of molecular weight 140 kDa was dissolved in the HAP suspension at 50°C for 4 h (Zhang *et al*., 1999). The solution was then ultrasonicated and rapidly transferred into a freezer at -20°C to solidify and induce solid-liquid phase separation. The solidified mixture was stored in freezer at 70°C for 2 h and then dioxane was removed using Christ Alpha 1-2 LD freeze dryer for 72 h (Zhang *et al*., 2006).

In order to make microcomposite scaffolds (mHAP/PLLA), hydroxyapatite with a mean diameter of 5 micrometer was purchased from Sigma (Cat No.

042138, Germany). mHAP/PLLA was then fabricated according to the procedure described for nHAP/PLLA composites.

PLLA powder (IMI, Canada) with molecular weight of 140 kDs was used to produce PLLA scaffolds. For this purpose, 0.05 g PLLA was dissolved in 100 ml dioxane to form 5% (wt) PLLA solution. The solution was then ultrasonicated and rapidly transferred into a freezer at -20°C to solidify and induce solid-liquid phase separation. The solidified mixture was kept at -75°C for 2 h and then freeze-dried for 72 h to remove dioxane. Nano-HAP/PLLA, micro-HAP/PLLA and PLLA scaffolds all had 50-170 micrometer pore sizes with about 90% porosity. The mechanical strength of nano-HAP/PLLA scaffolds was around 8.46 MPA and those of micro-HAP/PLLA and pure PLLA were about 4.61 and 1.79 MPA respectively.

Cell culture: This study was performed according to the procedure described elsewhere [19] and in strict adherence to protocols approved by Animal Care and Use Committee of Royan Institute (Tehran, Iran). Bone marrow was collected from the femurs of 10 Wistar rats with 8-10 weeks old. Separate cultures were established for each animal. About 10^6 bone marrow cells/ml were plated in 75-cm^2-culture flasks in the DMEM (Dulbecco's Modified Eagle Medium; Gibco, UK) supplemented with 15% FBS (fetal bovine serum, Gibco, UK), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco, UK). After 48 h, non-adherent cells were removed by medium replacement. The cultures were fed twice weekly for two weeks. By the time of confluency, the cells were lifted by tripsin/EDTA (Gibco) and split into two fresh 75-cm^2 flasks as a passaged-1 cells. With further successive subcultures, MSCs population was increased to a number sufficient to conduct the next stages of the experiment. To evaluate the osteogenic potential of the isolated cells, the medium of the passaged-3 culture was replaced by osteogenic DMEM medium containing 50 µg/ml ascorbic acid 2-phosphate (Sigma; USA), 10 nM dexamethasone (Sigma; USA) and 10 mM β-glycerol phosphate (Sigma; USA) for 21 days at the end of which the cells were fixed with 10% formalin for 10 min and stained with alizarin red (Sigma; USA) for 15 min (Eslaminejad *et al*., 2006 and 2007).

Three-dimensional cultures: Prior to culture initiation, the scaffolds were cut into small sizes of 5×5×2 mm dimension and sterilized by 70% ethanol for 30 min to remove the trace amount of dioxane. Due to the miscibility of dioxane and ethanol aqueous solution, the dioxane was extracted out and replaced with ethanol aqueous solution. After extraction, drying at room temperature was performed to remove all the remaining ethanol existing in the polymer matrix (Ho *et al*., 2004; Mohammadi *et al*., 2007; Budyanto *et al*., 2009). Before cell loading, the scaffolds were washed with PBS then, 5×10^5 passaged-3 MSCs were suspended in 50 µl DMEM medium and were placed on the top surfaces of the scaffold cubes located in a 12-well culture plate. Before the cultures were provided with the above medium, they were pre-incubated at 37ºC for 15 min during which the drop disappeared owing to its penetration into scaffold porosity. For proliferation assays, the cultures were provided with DMEM containing 15% FBS and antibiotics and for differentiation assays the cultures were provided with DMEM containing 50 µg/ml ascorbic acid 2-phosphate (Sigma; USA), 10 nM dexamethasone (Sigma, USA) and 10 mM β-glycerol phosphate (Sigma; USA), 100 IU/ml penicillin and 100 µg/ml streptomycin. All cultures were incubated in an atmosphere of 5% CO_2 at temperature of 37ºC.

Scanning Electron Microscopy (SEM): To examine the morphology of loaded cells within the scaffolds, the cell loaded scaffold specimens from day-7 culture were prepared for scanning electron microscopy (SEM) tests. To observe the interior of the composite, some cell-loaded scaffolds were sectioned in the middle with a razor blade. MSCs-loaded scaffolds were fixed in 2.5% glutaraldehyde solution at 4ºC for 24 h, followed by washing with PBS (phosphate buffer solution). The samples were then dehydrated sequentially with increasing concentration of ethanol (30%, 50%, 80% and 100%), coated with gold and visualized at an accelerating voltage using a Zeiss scanning electron microscope (Zeiss, DSM 940A, Germany).

Cell proliferation: Cell proliferation was analyzed by using [3-(4,5-dimethylthiazol-2-yl)-1,5-diphenyl tetrazulium bromide] (MTT, Sigma, USA) mitochondrial reaction. This assay was based on the ability of live cells to reduce a tetrazulium-based compound, MTT, to a purplish formazan product. Briefly, 5×10^5 cells were cultivated in composite scaffolds for a period of 1 week. Scaffold/cells constructs either from day 3 or 7 of 3D culture were then washed with PBS, transferred into new 24-well plates containing 5:1 ratio of media and MTT solution (5 mg/ml in PBS), respec-

tively and incubated for 2 h at 37ºC. After removing the solution, 0.5 ml of extraction solution (dimethylsulphoxide: DMSO) was added. The constructs were washed extensively by pipetting out repeatedly to allow total colour release. The absorbance of the supernatant was read with a microplate reader (BioTek EL x800, USA) at 540 nm. Cell number was determined through a standard curve that was established by using a known number of cells counted by a Neubauer-counting chamber. This was done according to Zund *et al*. who have found a linear correlation between the cell number and the MTT absorbency at 550 nm (Zund et al., 1999). A two-tailed student's t-test was used for comparing the results between the composite scaffolds under study.

Alkaline phosphates assay: To investigate the alkaline phosphatase activity (ALP) of the differentiating MSCs in osteogenic microenvironment of composite scaffolds, the cells in constructs were washed with PBS, homogenized with 1 ml Tris buffer (pH 7.4, Sigma) and sonicated. The cell lysate (0.1 ml) was mixed with 0.5 ml p-nitrophenyl phosphate (pNPP) substrate solution (Sigma) and 0.5 ml alkaline phosphate buffer solution (Sigma). After incubation at 37°C for 15 min, the above mixture was added with 10 ml of 0.05 N NaOH to stop the reaction and the absorption at 405 nm was measured spectrophotometrically. Total protein content was determined using Bradford method (1976) in aliquots of the same samples and calculated according to a series of gamma-globulin standards.

Mineralization of the cultures: The amount of alizarin red staining for osteogenic culture of the cells cultivated on composite scaffolds was quantified and compared using osteogenesis quantification kit (Chemicon, USA). This analysis was performed by determining OD405 values of a set of known alizarin red concentrations and comparing these values to those obtained from the osteogenic cultures. The procedure was conducted according to the manufacturer's instruction. In brief, the cultures were fixed in 10% formaldehyde for 15 min and followed by staining with alizarin red for 10 min. After washing five times with distilled water, the red matrix precipitate was solubilized in 10% acetic acid (Sigma,USA), and the optical density of solution was recorded at 405 nm with a microplate reader and compared to that of known alizarin red concentrations being provided by kit supplier. The scaffold without cells was used as the control to exclude the amount of calcium originating from scaffolds itself.

Semi-quantitative RT-PCR: The composites of cells/scaffold were minced into small pieces of less than 1 mm in dimensions. Total RNA was then isolated from the cells in culture using the Nucleospin RNAII kit (Macherey-Nagel, Germany) according to the manufacturer's specifications. The RNA samples

Table 1. Primers used in RT-PCR.

	Gene name	Direction	Sequence	AT	Product size	PCR Cycle
Osteogenic genes	Collagen type I	Forward	F: 5'GAA TAT GTA TCA CCA GAC GCA G 3'	57	186	33
		Reverse	R: 5' AGC AAA GTT TCC TCC AAG AC 3'			
	Osteocalcin	Forward	F: 5' GGA GGG CAG TAA GGT GGT G 3'	54	293	33
		Reverse	R: 5' GCT GTG CCG TCC ATA CTT TC3'			
	Runx2	Forward	F: 5' CAG TTC CCA GGC ATT TCA TC 3'	55	367	33
		Reverse	R: 5' CAG CGT CAA CAC CATCAT TC 3'			
Housekeeping	GAPDH	Forward	F: 5' TGC TGA GTA TGT CGT GGA GTC 3'	56	611	30
		Reverse	R: 5' AAA GGT GGA AGA ATG GGA G 3'			

were digested with DNase I (EN0521; Fermentas) to remove contaminating genomic DNA, and then quantified spectrophotometrically at 260 nm. All RNA isolates had an OD260:OD280 between 1.8 and 2.0, indicating clean RNA isolates. A two-step semi-quantitative RT-PCR method was used to measure the specific gene expression during osteogenic differentiation of mesenchymal stem cells. Standard RT was performed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (K1622; Ferments, Germany) and random hexamer primer was used as a primer in the first step of cDNA synthesis. Relative RT-PCR was performed to measure expression of rat osteocalcin, RunX2 and COL I genes. Primer sequences and optimal PCR annealing temperatures (AT) are listed in Table 1. Polymerase chain reactions were performed on a PTC-200 PCR machine (MJ Research Inc, MA, USA) using 2 µl cDNA, 1×PCR buffer (AMSTM; CinnaGen Co., Tehran , Iran), 200 µM dNTPs, 0.5 µM of each of the forward and reverse primers and 1U Taq DNA polymerase (Fermentas, MD, USA). PCR reactions were performed on a Mastercycler gradient machine (Eppendorf, Germany). Amplification conditions involved the initial denaturation at 94°C for 5 min followed by 35 cycles (25 cycles for GAPDH) of denaturation at 94°C for 45 s, annealing for 45 s, extension at 72°C for 30 s, and a final polymerization at 72°C for 10 min. Each PCR was performed under linear conditions with GAPDH used as an internal standard. Amplified DNA fragments were electrophoresed on 1.5% agarose gel. The gels were stained with ethidium bromide (0.5 mg/ml) and photographed on a UV transilluminator (Uvidoc, UK). The gel images were digitally captured with a CCD camera and analyzed using the UVI band map program (Uvitec, Cambridge, UK). For the semi-quantitative determination of mRNA levels of the candidate genes, transcript levels were normalized to the corresponding GAPDH. Data were analyzed by SPSS 13.0 for windows.

Statistical analysis: Mean values ± SD (standard deviation) were calculated for the data obtained from the MTT assay, mineralization quantification, ALP assay and RT-PCR analysis. The data were analyzed with ANOVA using SPSS software version 13. A P value < 0.05 was considered to be significant.

RESULTS

Cell Culture: The cultures of bone marrow cells were regularly observed with a phase contrast invert microscope. According to these observations, some marrow cells formed a number of colonies consisting of a few adherent fibroblastic cells (Fig. 1A). The colonies grew larger as the time was advanced. Ten days after culture initiation, all the available surfaces of the culture dishes were covered with fibroblastic cells (Fig. 1B). Passaged-3 fibroblastic cells tended to differentiate into bone cells in monolayer cultures (Fig. 1C).

SEM: Cell attachment was observed using SEM. The MSCs seeded on scaffolds surfaces appeared as spherical in morphology. Cell density on nano-HAP/PLLA scaffolds (Figs. 2C and 2D) appeared to be higher than those on either micro-HAP/PLLA (Figs. 2E and 2F) or pure PLLA (Fig. 2A and 2B) scaffolds.

Cell proliferation: According to our results, within the microenvironment provided by nano-HAP-PLLA

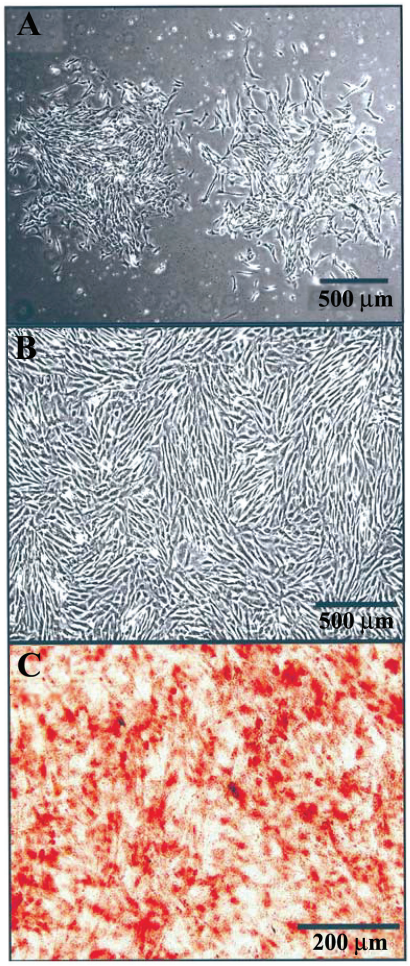

Figure 1. Rat bone marrow cultures:A: Four days after culture initiation a number of colonies consisting of a few fibroblastic cells appeared at primary cultures; B: the colonies grew and formed a monolayer; C: alizarin red staining of passaged-3 mesenchymal stem cells indicating of the cell ability to differentiate into a mineralized-matrix producing cells.

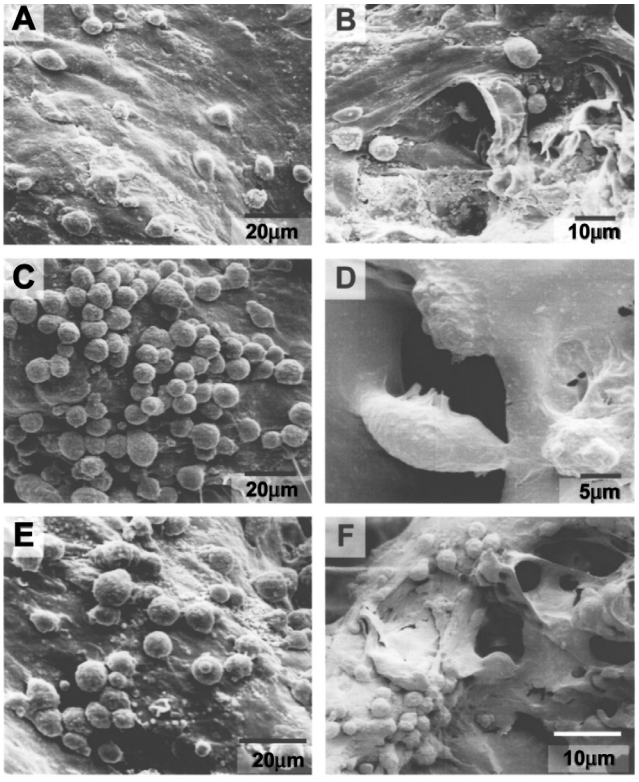

Figure 2. Scanning electron microscopy of 3D cultures. A-B: MSCs on pure PLLA, C-D: nano-HAP/PLLA and E-F: micro-HAP/PLLA surfaces. The cells appeared as spherical shape. Cellular density seems to be much higher on nano-composite scaffold.

scaffold, MSCs tended to proliferate more than that of micro-HAP/PLLA and pure PLLA. MTT results indicated that the scaffolds are compatible with cell proliferation. Comparatively in nano-scaffolds, there appeared to be significantly more cell proliferation than the macro-HAP/PLLA and PLLA alone ($P < 0.05$). On day 7, MSCs population in nano-scaffolds reached to a value of $5.76\times10^5 \pm 0.1\times10^5$/scaffold, while at this time the number of the cells in micro-scaffolds were $3.92\times10^5 \pm 0.1\times10^5$/scaffold (Fig. 3). The number of the cells in pure PLLA was about $4.17\times10^5 \pm 0.1\times10^5$/scaffolds. All differences were statistically significant ($P < 0.05$).

ALP assay: ALP activity is a biochemical marker for osteoblastic phenotype, bone mineralization and differentiation. According to our results, the ALP activity tended to be significantly increased in nano-scaffolds compared to those in micro-HAP/PLLA and pure scaffolds particularly on day 21 of osteogenic culture period. ALP absorbance value for nano-scaffolds was 0.85 ± 0.02 versus 0.72 ± 0.02 and 0.71± 0.02 for micro-

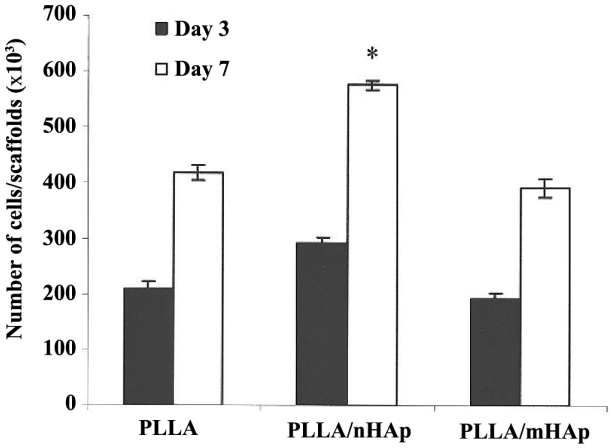

Figure 3. Cell proliferation (MTT assay) on 3D cultures. The number of MSCS was significantly increased on nano-HAP/PLLA scaffolds. (*) statistical difference with $P< 0.05$.

scaffolds and pure PLLA, respectively (Fig. 4A). The differences were statistically significant ($P < 0.05$).

Mineralization: Deposition of mineralized matrix is considered to be as a significant event during osteoblast differentiation which endows the tissue its characteristic strength and stability. Among the microenvironment of studied composite scaffolds and pure PLLA, the amount of mineralized matrix production tended to be significantly high in nano-HAP/PLLA scaffolds (0.946 ± 0.02 mM). The scaffold possessed a significant difference compared with micro-HAP/PLLA and pure PLLA scaffolds which presented mineralization values of 0.717 ± 0.03 and 0.721 ± 0.05 mM, respectively (Fig. 4B).

Relative gene expression: To thoroughly examine and determine the level of bone differentiation in the scaffolds, semi-quantitative analysis was performed to determine the relative expression of bone gene markers in each 3D culture. According to semi- quantitative RT-PCR analyses, the expression level of collagen type I (Fig. 5A), osteocalcine (Fig. 5B) and RunX2 (Fig. 5C) genes were relatively higher in the cells cultivated on nano-scaffolds compared to those cultivated in either micro-HAP/PLLA or pure PLLA ($P < 0.05$).

DISCUSSION

In this study passaged-3 rat MSCs were used to establish 3D cultures on three different scaffolds including nano-HAP/PLLA, micro-HAP/PLLA and pure PLLA

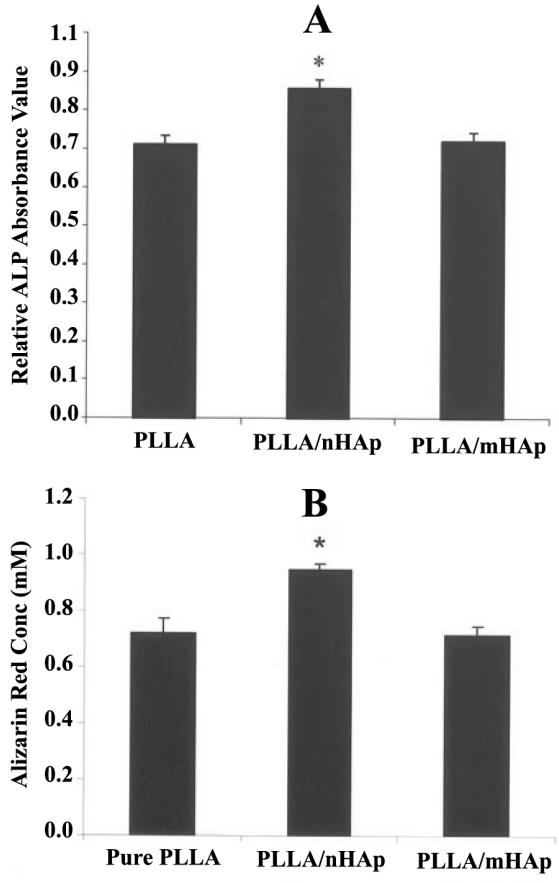

Figure 4. A: Comparison of the alkaline phosphatase (ALP) activity for the cells in different scaffolds; this activity tends to be significantly increased in the cells cultivated on nano-scaffolds compared to those of the cells seeded in micro-HAP/PLLA and pure PLLA scaffolds. B: A graph demonstrating the alizarin red concentrations in different 3D cultures. (*) statistical difference with P< 0.05.

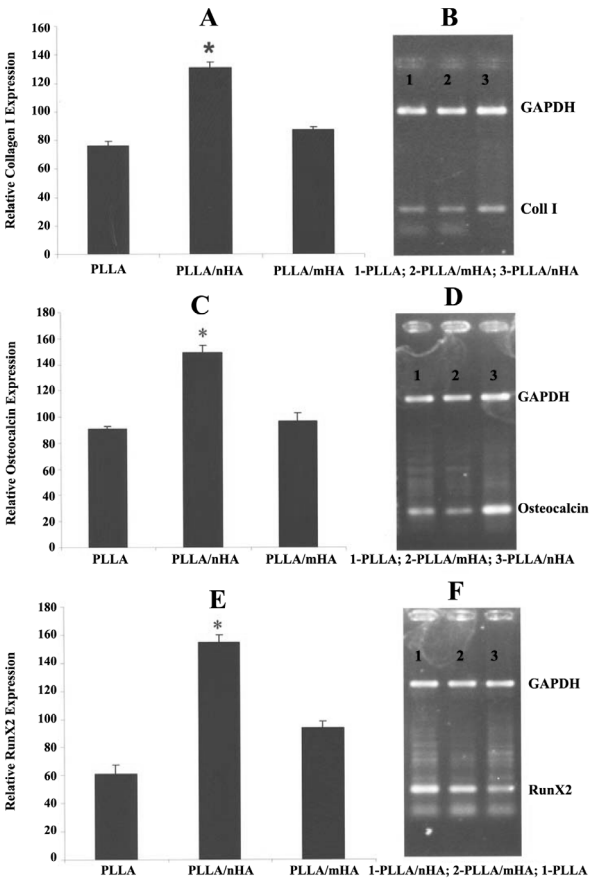

Figure 5. Expression of bone specific genes by semi-quantitative RT-PCR. A: the graph and B: the corresponding PCR gel image indicating collagen I expression. C: the graph and D: the corresponding PCR gel image showing osteocalcin expression. E: the graph and F) the corresponding PCR gel image demonstrating RunX2 expression. (*) statistical difference with P< 0.05. PLLA: poly(*l*-lactic acid); nHA: nano-hydroxyapatite; mHA: micro-hydroxyapatite.

scaffolds. These scaffolds have recently been developed at our laboratory (Nejati *et al*., 2009). The objective of this study was to determine which scaffold microenvironment would be suitable for MSCs proliferation and bone differentiation.

Cell attachment on scaffold's surfaces is the prerequisite of their proliferation and differentiation. The behavior of cells on biomaterial depends on scaffold's surface characteristic including roughness, topology, damp absorbance, electrical charge and chemistry and energy of the surfaces. These parameters have significant impact on conformation, orientation and quality of adherent protein-like vitronectin and fibronectin as well (Verrier *et al*., 2004; Anselme *et al*., 2000; García *et al*., 1999). In this study cell attachment and proliferation were determined using histological observations and MTT assays respectively. Our results indicated that the surfaces of the scaffolds were compatible with MSC attachment and proliferation. In this regard, the surfaces provided by nHAP/PLLA scaffolds appeared to be more appropriate than those provided by the other scaffolds. This would be due to the specific topology of HAP particles in nano-dimension in nHAP/PLLA scaffolds. Indeed when scaffolds were prepared using HAP in nano-dimensions, comparatively more extensive surface area was created compared to the micro-dimensional HAP scaffolds. Therefore, extensive surface area provides more MSCs attachment in the internal spaces of loaded scaffolds.

While there were 5×10^5 cells/scaffold to initiate the 3D cultures, the number of the cells appeared to be decreased on day 3 in all studied groups (Fig. 3). The decreased number of cells could be due to the failure of some cells in participating and establishing the 3D

culture. Since the cells that were being used for 3D cultures were the passaged 3 MSCs prepared by trypsinization of passage 2 cultures, which may have damaged the cell surfaces affecting the cell ability to survive in 3D cultures. Furthermore, at 3D culture initiation, for MSC loading onto scaffold, the cells were suspended in 50 µl DMEM medium and placed on the top surface of the scaffolds. Under this condition the cells started to penetrate the scaffolds pore system by the action of gravity. Since the scaffolds were highly porous with a relatively large pore size (50-170 micrometer), some cells more likely passed through the scaffold and left on the other side of the scaffolds.

It is important to emphasize that there is a high cell number on nano-scaffold compared to the micro-scaffold as well as pure PLLA at the end of cultivation period. There are two explanations; first, the nano-scaffolds provided comparatively more surface area for cell attachment which was followed by more cells adhered on the scaffold internal spaces. Adhered cells then survived and proliferated. Secondly, the surface-of nano-HAP/PLLA scaffolds may have had mitogenic effect on MSCs resulting in more cells housed within the nano-scaffolds **as** compared to the two other scaffolds. The degree of contribution of each possibility remains to be investigated.

Differentiation level of the cells on surface of the constructs would be of significant importance for regenerative medicine. In general, there could be two strategies with respect to the use of MSCs-mediated tissue regeneration. One route would be to use the cells as undifferentiated state, allowing them to undergo differentiation in defect sites. The shortcoming of this approach is the differentiation of unwanted cells other than the desired cells which may occur in transplantation site. For instance, if the cells are desired to regenerate bone tissue, other cells may be produced in the site by unwanted differentiation. The alternative approach is to fully differentiate the cells into the desired cells prior to their transplantation (Weissman *et al*., 2000). Such strategy could indeed guarantee the administration of the intrinsic differentiated cells into the repair site. This strategy may clarify the significance of MSCs *in vitro* differentiation into bone tissue prior their use in regenerative medicine. In the present study, bone differentiation of marrow-derived MSCs was quantified on three different 3D scaffolds to determine which one can provide appropriate microenvironment for stronger differentiation. Based on these results it was shown that the Nano-scaffolds were according to our results provided more suitable microenvironment for MSCs bone differentiation than either micro- scaffolds or pure PLLA scaffolds since the ALP activity as well as the level of bone specific gene expression of the MSCs were significantly higher in nano-scaffolds compared to two other examined scaffolds. This could be due to large surface area provided by nano-hydroxyapatite particles compared to micro- hydroxyapatite particles. The better performance of hydroxyapatite/PLLA scaffolds in comparison with the pure PLLA would be attributed to hydroxyapatite as a natural constituent of bone tissue.

CONCLUSIONS

This study indicated that nano-HAP/PLLA scaffolds provide a microenvironment in which MSCs can undergo much proliferation compared to the surfaces of micro-HAP/PLLA and pure PLLA scaffolds. Furthermore, it seems that microenvironment of nano-surfaces are more appropriate for MSCs bone differentiation. Given these data, a construct consisting of nano-HAP/PLLA scaffold loaded with MSCs would be an appropriate bone substitute for use in regenerative medicine.

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