Original Article

Study of Mesenchymal Stem Cell Proliferation and Bone Differentiation on Composite Scaffolds of PLLA and Nano Hydroxyapatite with Different Morphologies

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Abstract

Objective: Nowadays, bone constructs elaborated according to tissue engineering principles are being regarded as an ideal choice for the reconstruction of segmental bone defects. In this study, proliferation and bone differentiation of marrow-derived mesenchymal stem cells (MSCs) were compared in different composite scaffolds containing varying morphologies of nano hydroxyapatite (nHAP).

Materials and Methods: Needle nHAP/PLLA (poly (L-lactide acid)), spherical nHAP/PLLA and rod nHAP/PLLA scaffolds were prepared and 3D cultures of passaged-3 rat MSCs were established using the scaffolds. The loading of the cells onto the scaffold internal spaces was confirmed with microscopy and their proliferation was determined by MTT assay. To compare the osteogenic differentiation of the cells on the scaffold surfaces, osteogenic 3D cultures were established and kept for 21 days. At the end of this period culture mineralization and relative bone-related gene expression were quantified using the alizarin red quantification assay and semi quantitative RT-PCR analysis respectively. ANOVA was used to compare the data.

Results: According to the MTT assays, cells adhered to all the studied scaffold surfaces tended to proliferate. In this respect the microenvironment provided by the needle nHAP/PLLA appeared much better than that of either the spherical or rod nHAP/PLLA scaffolds ($P<0.05$). Similarly, mineralization was observed to be heavier for the needle nHAP/PLLA scaffold compared to the two other composite scaffolds. In addition, the relative expression of coll I, osteocalcin, runx2 and ALP genes all appeared to be significantly higher in the cells cultivated on needle nHAP/PLLA scaffolds versus their spherical and rod counterparts.

Conclusion: Overall, needle nHAP/PLLA scaffolds appear to provide the most appropriate matrix for producing bone construct using MSCs.

Keywords: Hydroxyapatite, Scaffold, Mesenchymal Stem Cell, Osteogenesis, Cell Proliferation

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Introduction

Bone associated problems are considered to be an economic and social problem in the field of modern medicine. The majority of bone tissue defects repairs spontaneously or needs minimal intervention. In some circumstances, such as injuries following a car accident, congenital cleft palate, lesions left by the removal of bone cancer and periodontal disorders, the defects are only partially repaired due either to the size of the defect or the slow regeneration process inherent in the etiology of the defect (1).

Multiple repair strategies including autograft, allograft and use of metal, have so far been proposed to overcome these problems. Autograft transplantation is considered the best option since it is obtained from the patient's own body and brings osteogenic, osteoinductive and osteoconductive components into the defect sites. Nevertheless, since obtaining autograft bone is quantitatively limited and requires an extensive operation, which may causes morbidity, pain and possible infection of the donor site, a search for alternative substitutes has emerged (2-4). Allograft bone transplantation would be an alternative choice that does not exhibit the limitations associated with autograft bone since it can be obtained from cadaver tissue. Allograft disadvantages, however, include less osteoinductivity, possible triggering

of the host immune response and likely transmission of disease (5). Other choices would be the use of metal implants. Although these substitutes are applicable in some cases they possess several disadvantages. These include first, they do not degrade after implantation, hence, secondary surgery is necessary to remove them from the host, and second, they may release toxic ions that trigger host immune response and infection (6, 7). Given these data, nowadays, bone constructed using tissue engineering methods is considered a valuable substitute for bone grafts.

Tissue engineering is a multidisciplinary science, defined as the application of the principles and methods of life and engineering sciences in order to understand the relationships between structure and function in normal and injured mammalian tissues and to develop biological substitutes for repair, maintenance and improvement of tissue function (8). One purpose of tissue engineering is to synthesize scaffolds that can be used as a substitute for the tissue matrix and help the new tissue to grow. Scaffolds should be biocompatible, biodegradable and possess appropriate mechanical properties as well as suitable surfaces. Moreover they should provide a microenvironment in which the cells can proliferate and differentiate into desired cell lineages (9-11).

Polylactic acids are among the polymers most frequently used in bone tissue engineering. These materials possess an appropriate degradation rate but their surfaces are not suitable for cell attachment. On the other hand, Hydroxyapatite and tricalcium phosphate, which are indeed the main components of the mineralized matrix of natural bone, are being considered as a suitable matrices for producing bone construct. The main pitfall associated with these matrices has been reported to be their fragile consistency. However, the combination of poly (L-lactide acid) and Hydroxyapatite reduces the limitation associated with each scaffold alone (12-18). The other parameter is the dimensions of the materials that are used to produce the scaffolds. In this regard the use of hydroxyl-apatite in nano dimensions has been shown to possess a valuable advantage over its micro counterpart (19-20).

In addition to the scaffolds, the other key component in designing bone construct is the cells that need to be cultivated three dimensionally on the scaffold surfaces. Up to now, osteoblastic, as well as mesenchymal stem cells (MSCs), have gained fairly extensive attention as cellular candidates in bone tissue engineering (21). 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; extensive self-renewal ability, and the potential for multilineage differentiation that make them more suitable for tissue engineering applications. Moreover, these cells can easily be isolated from bone marrow aspirate, which involves inserting a needle into the iliac crest, thus involving relatively less morbidity than the either bone or periosteum harvest (22-29).

In a previous study, we manufactured and introduced composite scaffolds consisting of poly (L-lactide acid) and needle-like nano hydroxyapatite (19). In the present study, composite scaffolds containing different morphology of hydroxyapatite including needle, spherical and rod were prepared and compared in terms of their appropriateness for producing bone constructs using marrow-derived MSCs. For these purposes the proliferation and bone differentiation of marrow-derived MSCs were determined and compared in 3D cultures on the scaffolds.

Materials and Methods

Preparation of the scaffolds

The scaffolds were made according to the method described in our previous paper (19). Briefly, the nHAP was prepared by a 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. 0.03 M aqueous solution of $(NH_4)_2HPO_4$ was added drop wise to a 0.05M aqueous solution of Ca $(NO_3)_2.4H_2O$ while stirring to form nHAP particulates. In all experiments the pH of the solution was adjusted to 11 using diluted NaOH solution (Merck, Germany) and the temperature was maintained at 70 ± 5°C (needle morphology) or 80 ± 5 (rod morphology) or 40 ± 5 (spherical morphology). The precipitated HAP particles were aged for 24 hours at room temperature. The resulting product was centrifuged at 9000 rpm for 30 minutes using a sigma-12151 rotor. The morphology of the synthesized nHAP was confirmed by scanning electron microscopy (SEM). For this purpose the samples were coated with gold before examination by SEM (Fig 1A-C). Particles with needle morphology tended to have an average size of 250×45nm, spherical particles to have an average size of 50 nm and the particles with rod morphology had an average size of 290×62 nm.

nHAP/PLLA composites were made using the following procedure: synthesized nHAP powder was dispersed in 10 cc 1, 4 dioxane (Merck, Germany) by sonication for 2 minutes at 130 W (Bandelin sonoplus HD2200, Berlin, Germany). Then 0.5g PLLA (IMI, Canada) of molecular weight 140 KDa was dissolved in the HAP suspension for 4 hours. 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 maintained at that temperature for 2 hours and then transferred into -75°C and 0.03 mbar freeze-dryer vessels for 72 hours to remove dioxane.

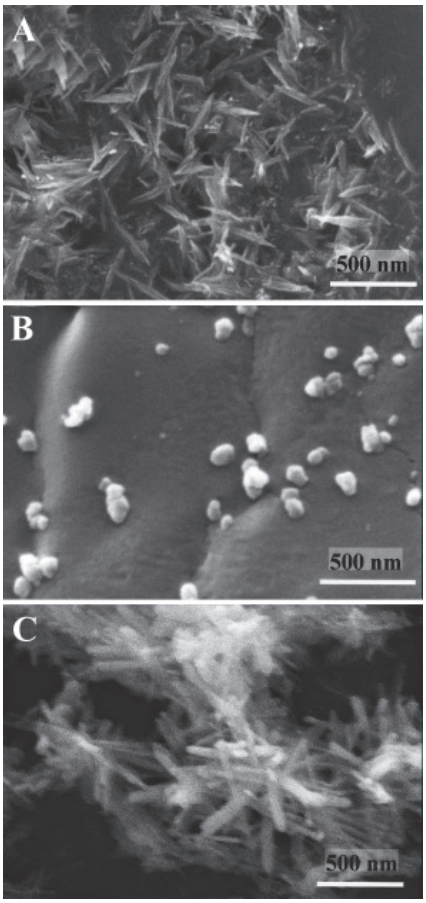

Fig 1: Representative scanning electron microscopic image of the morphology of synthesized nano HAP particles. A. Needle morphology, B. Spherical morphology C. Rod morphology.

Cell culture

This study was performed with strict adherence to protocols approved by the Animal Care and Use Committee of the Royan Institute (Tehran, Iran). Bone marrow was collected from the femurs of 10 Wistar rats 8 to 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 hours, non-adherent cells were removed by medium replacement. The cultures were fed twice weekly for two weeks. When confluent 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, the MSC population was increased to a number sufficient to conduct the next stages of the experiment.

Differentiation potential

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), 10nM dexamethasone (Sigma; USA) and 10 mM ß-glycerol phosphate (Sigma; USA) for 21 days at the end of which the cells were fixed with 10% formalin for 10 minutes and stained with alizarin red (Sigma; USA) for 15 minutes. For adipogenic differentiation, confluent passaged-3 cells cultivated in 6-well culture plates were provided with adipogenic DMEM medium containing 100 nM dexamethazone (Sigma, USA) and 50 mg/ml indomethasine (Sigma, USA). The cultures were then incubated for 21 days at the end of which the culture was fixed with 4% formalin at room temperature, washed by 70% ethanol and then stained by oil red solution in 99% isopropanol for 15 minutes. After the dye solution was removed the cultures were washed with 70% ethanol and observed by light microscopy.

Three dimensional cultures

Prior to culture initiation, the scaffolds were cut into small pieces 5×5×2 mm in dimension and sterilized using 70% ethanol for 30 minutes. Then, 5×10^5 passaged-3 MSCs were suspended in 50µl DMEM medium and placed on the top surfaces of the scaffold cubes located in the wells of 12-well culture plates. Before the cultures were provided with medium they were pre-incubated at 37ºC for 15 minutes during which the drop disappeared due to its penetration into the porous scaffold. 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 ß-glycerol phosphate (Sigma; USA), 100 IU/ml penicillin and 100µg/ml streptomycin. All cultures were incubated in an atmosphere of 5% CO_2 and a temperature of 37ºC.

Light microscopy

To observe the cells within the scaffold, cell-scaffold constructs were prepared for light microscopy observation at the end of culture period. For this purpose, the cultures were fixed with 10% formalin, dehydrated in ascending ethanol and embedded in paraffin.

The 6-micrometer paraffin serial sections were then made and stained by H&E.

Cell proliferation

Cell proliferation was analyzed using [3-(4, 5-dimethylthiazol-2-yl)-1, 5-diphenyl tetrazulium bromide] (MTT, Sigma, USA) mitochondrial reaction. This assay is 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. Cells/scaffold constructs either from day 3 or 7 of 3D culture were then washed with PBS, transferred into new 24-well plates containing a 5:1 ratio of media and MTT solution (5 mg/mL in PBS), respectively and incubated for 2h at 37ºC. After removing the culture media, 0.5mL of extraction solution (dimethylsulphoxide: DMSO) was added. The constructs were washed extensively by pipetting up and down repeatedly to allow total color release. The absorbance of the supernatant was read with a microplate reader (BioTek EL x800, USA) at 540 nm. The size of the cell population was determined via a standard curve that was established using a known number of cells counted by a coulter counter. A two-tailed student's t-test was used for comparing the results between the studied composite scaffolds.

Mineralization of the cultures

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

Semi quantitative Revers-Transcription Polymerase chain reaction

The cells/scaffold composites were minced into small pieces of less than 1 mm in dimension. 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 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 the primer in the first step of cDNA synthesis. Relative RT-PCR was performed to measure the expression of rat osteocalcin, Alp, 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 2µl cDNA, 1×PCR buffer (AMS™; CinnaGen Co., Tehran, Iran), 200 µM dNTPs, 0.5µM of each of 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 consisted of initial denaturation, 94°C for 5 minutes, followed by 35 cycles (25 cycles for GAPDH) of denaturation at 94°C for 45 seconds, annealing for 45seconds, extension at 72°C for 30 seconds, and a final polymerization at 72°C for 10 minutes. 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 (0.5mg/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 semi-quantitative determination of mRNA levels of the candidate genes, transcript levels were normalized to the corresponding GAPDH.

Statistical analysis

Mean values ± SD were calculated for the data obtained from the MTT assay, mineralization quantification, 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

Marrow cells were observed to grow with forma-

tion of fibroblastic colonies in the primary cultures. Some small cells were also observed scattered among the fibroblastic colonies (Fig 2A).
The culture was dominated by mesenchymal fibroblastic cells after several subcultures. Passaged-3 cultures appeared to be composed of homogenously uniform fibroblastic cells (Fig 2B).

Differentiation potential

The osteogenic cultures stained positively with alizarin red indicating that the culture undergoes mineralization following osteogenic differentiation (Fig 2C). In the adipogenic cultures, lipid droplets were observed in differentiating cells several days after culture initiation. These were identified using oil red staining (Fig 2D). The osteogenic and adipogenic potential of the cells indicated that the isolated cells were MSCs.

Cells within the scaffolds

Hematoxilin and eosin staining of the sections prepared from the scaffolds indicated that cells had entered the scaffold's internal pore system and had covered the surfaces (Fig 3A-C).

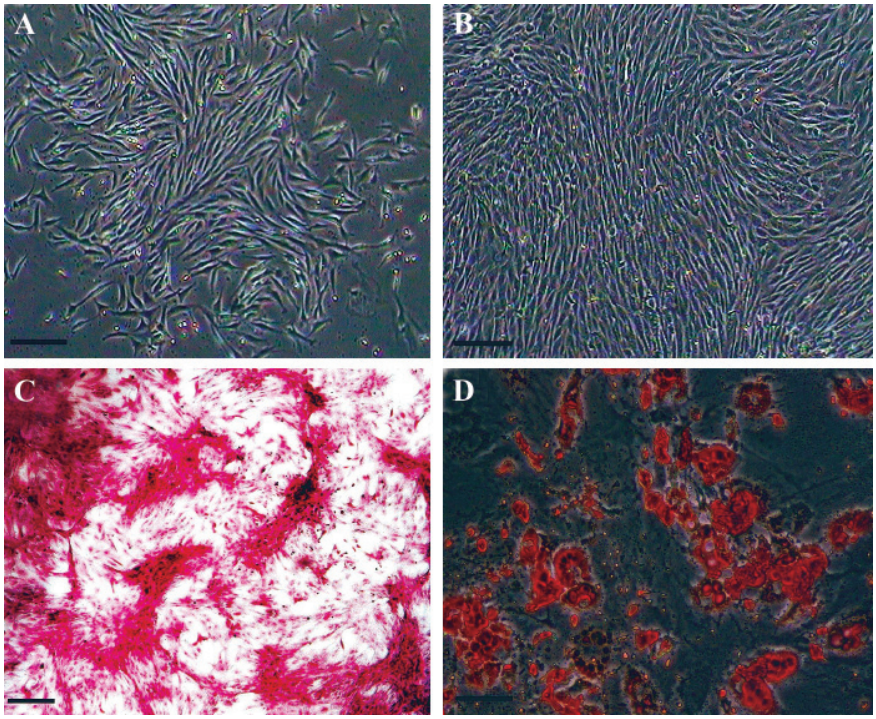

Fig.2: Marrow cell culture. A) Marrow cell at primary culture (Bar=100µm). B) Marrow cell at passage 3 (Bar=100µm). C) Osteogenic culture of marrow passaged-3 culture (Bar=500µm). D) Adipogenic culture of marrow passaged-3 cells (Bar=100µm).

Table 1: Primers used in RT-PCR analysis

	Gene name	Direction	Sequence	AT	Product size
Osteogenic genes	Collagen type I	Forward	F: 5'GAA TAT GTA TCA CCA GAC GCA G 3'	57	186
		Reverse	R: 5' ABC AAA GTT TCC TCC AAG AC 3'		
	Alp	Forward	F: 5' ACA CGG ACA AGA AGC CCT 3'	59	203
		Reverse	R: 5' GTG AAG CAG GTG AGCCAT AG 3'		
	Osteocalcin	Forward	F: 5' GGA GGG CAG TAA GGT GGT G 3'	54	293
		Reverse	R: 5' GCT GTG CCG TGG ATA CTT TC3'		
	Runx2	Forward	F: 5' CAG TTC CCA GGA ATT TCA TC 3'	55	367
		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
		Reverse	R: 5' AAA GGT GGA AGA ATG GGA G 3'		

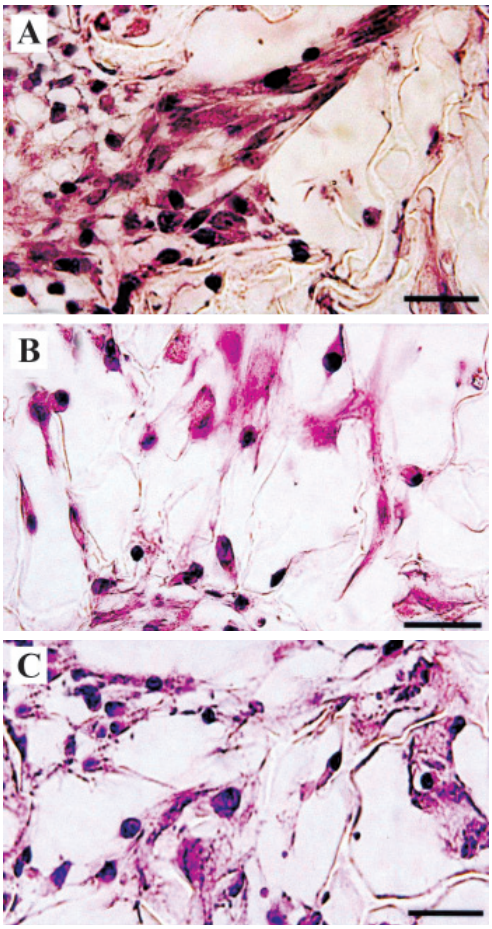

Fig 3: Representative photomicrograph of passaged-3 marrow derived mesenchymal stem cell seeded composite scaffolds. A) Needle nHAP/PLLA B) Spherical nHAP/PLLA C) Rod nHAP/PLLA. (Bar= 50 µm).

Viability and proliferation of the cells

MTT results indicated that all scaffolds were compatible with cell proliferation. Comparatively in the composite scaffolds with needle nHAP, there appeared to be significantly more cell proliferation than those either with rod or spherical nHAP ($p<0.05$). On day 7 the MSC population of the scaffolds with needle nHAP had reached 596.67×10^3. At the same time the numbers of cells in the scaffolds with rod and spherical nHAP were $426.67\times10^3\pm$ and 421.33×10^3 per scaffold respectively (Fig 4A) ($p<0.05$).

Culture mineralization

According to the alizarin red staining quantification assays composite scaffolds with needle nHAP demonstrated significantly heavier calcium precipitation (1.052±0.0232 mM) compared to composite scaffolds with either rod (0.924±0.03213 mM) or spherical (0.768±0.0544 mM) morphology (Fig 4B) ($p<0.05$).

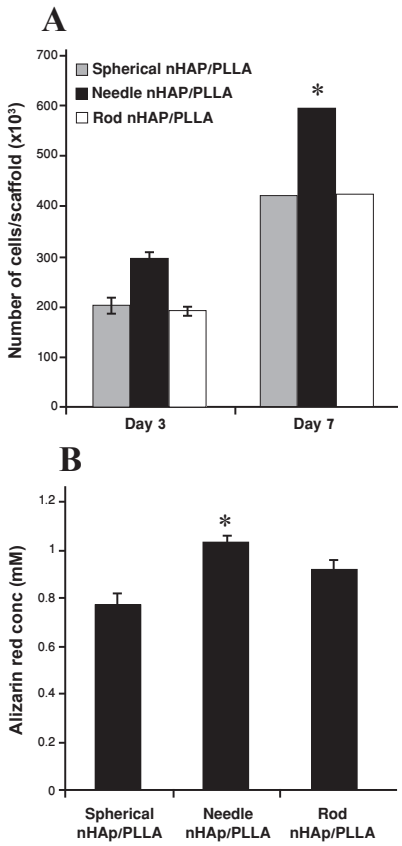

*Fig 4: A. MSC proliferation in the composite scaffolds. The proliferation was extensive in the needle nHAP/PLLA microenvironment. * indicates a statistically significant difference ($p<0.05$). B. Culture mineralization. Similarly in the needle nHAP/PLLA culture there was heavier mineralization. * indicates a statistically significant difference ($p<0.05$)*

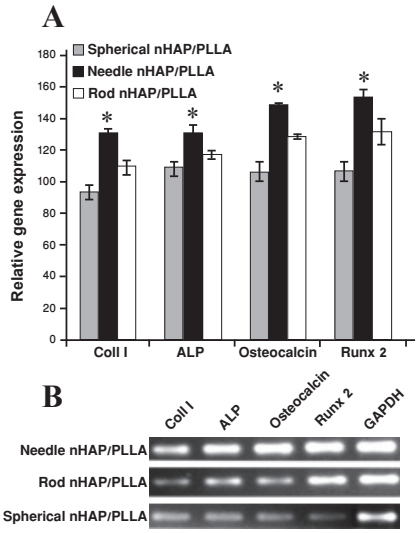

*Fig 5: A. Relative osteogenic gene expression on different composite scaffolds. The needle nHAP/PLLA scaffold tended to provide the most appropriate microenvironment evidenced by relatively higher expression of bone-related genes. * indicates a statistically significant difference ($p<0.05$). B. Gel image of osteogenic genes.*

Gene expression

The relative expression of collagen type I, osteocalcin, Runx2 and ALP genes was higher in the cells cultivated on composite scaffolds with needle nHAP morphology. The difference was statistically significant compared to those with either rod or spherical nHAP morphologies (Fig 5A-B).

Discussion

In this study, proliferation and bone differentiation of marrow-derived MSCs were quantified and compared in 3D cultures on needle nHAP/PLLA, rod nHAP/PLLA and spherical nHAP/PLLA. According to our results, the scaffolds with needle nHAP/PLLA appeared to be more appropriate for bone construct manufacture since cell density, mineralization and bone related-gene expression tended to be more extensive in these scaffolds compared to those containing either spherical nHAP/PLLA or rod nHAP/PLLA. Studies like this help to develop suitable 3D bone constructs for use in the repair of large bone defects.

The most significant points related to polymers are their biocompatibility and surface characteristics in relation to cell adherence. Cellular attachment is the prerequisite for proliferation and differentiation. The behavior of cells on biomaterial is dependent on surface characteristics including roughness, topology, damp absorbance, electrical charge, chemistry and energy. These parameters have significant impact on the conformation, orientation and quality of adherent protein like vitronectin and fibronectin, which are the mediators of cell/biomaterial interactions (30-33). In this study, cell attachment and proliferation were determined using histological and MTT assays respectively. Our results indicated that the surfaces of the scaffolds were compatible with MSC attachment and proliferation. In this respect, surfaces with needle nHAP/PLLA scaffolds appeared to be more appropriate for cell attachment and their subsequent proliferation. All these properties can be attributed to the combined presence of nHAP/PLLA and needle morphology.

According to our results, within the microenvironment provided by the needle nHAP/PLLA scaffold, MSCs tended to proliferate more than the microenviroments provided by either rod or spherical nHAP/PLLA. While about 5×10^5 cells were, in the present study, used to initiate the 3D cultures, the number of the cells in some cultures appeared to be decreased at the end of the cultivation period reaching 421.33×10^3 and 426.67×10^3 (for the spherical nHAP/PLLA and rod nHAP/PLLA respectively). One potential explanation for this is that during the cultivation period some cells come out of the scaffold before they can create an attachment to the scaffold's surfaces. This means that two factors probably contribute to the presence of a high number of MSCs in needle nHAP/PLLA scaffolds. First, the scaffold surface was more appropriate for cell attachment as a result of which more cells remained attached and survived. Second, the surfaces of needle nHAP/PLLA scaffolds may have a mitogenic effect on MSCs, resulting in the presence of more cells within this scaffold compared to the two other scaffolds. The relative contribution of each factor remains to be investigated.

In manufactured bone construct designed to be used for the repair of bone defects one of the most important parameter for repair success is the level of differentiation of the cells. In general, there could be two strategies with respect to the use of MSC-mediated tissue regeneration. One route would be to use the cells in an undifferentiated state, allowing them to differentiate in the in vivo microenvironment. The disadvantage of this approach is that unwanted differentiation of cell types, other than those desired, may occur at the transplantation site. Therefore the alternative approach is to fully differentiate the cells down the osteoblast pathway prior to their transplantation (34). Such a strategy could guarantee the administration of suitably differentiated cells into the repair site.

In this study, bone differentiation was quantitatively examined by determining the amount of matrix mineralization as well as the relative expression of some bone-related genes. One important characteristic of bone matrix is the degree of mineralization. Alizarin red is able to form a complex with deposited mineralized matrix. The results obtained by our assays indicated that mineralization occurred in all 3D cultures, but that there appeared to be more mineralization in needle nHAP/PLLA scaffolds than rod and spherical nHAP/PLLA scaffolds. The high level of calcium deposition in the needle nHAP/PLLA scaffolds is one of the most important findings to come out of the present investigation. What mechanism lies behind this property is at present unknown and therefore requires further investigation.

To thoroughly examine 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 these analyses, the relative expression of collagen type I, osteocalcine, ALP and RunX2 genes was high in the cells cultivated on needle nHAP/PLLA scaffolds compared to those cultivated on either rod nHAP/PLLA or spherical nHAP/PLLA scaffolds. The reason why the needle-like morphology was better than the rod and spherical morphol-

ogy is not known and needs further investigation.

Conclusion

Taken together, composite scaffolds containing needle HAP tended to be better for the construction of bone than scaffolds containing either rod or spherical HAP. The microenvironment provided by the needle HAP scaffolds was also more conducive to MSC proliferation than those of either rod or spherical HAP scaffolds. These data suggest that the needle HAP/PLLA scaffold is the most suitable matrix for the construction of bone using MSCs.

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