Original Article

In vivo Bone Formation by Canine Mesenchymal Stem Cells Loaded onto HA/TCP Scaffolds: Qualitative and Quantitative Analysis

Mohamadreza Baghaban Eslaminejad, Ph.D.[1]*, Mohammad Jafarian, D.M.D., M.Sc.[2], Arash Khojasteh, D.M.D.[2], Fatemeh Mashhadi Abbas,D.D.s., M.Sc.[2], Mohammad Mehdi Dehghan, D.V.Sc.[3], Rahele Hassanizadeh, D.D.S.[4]

1. Stem Cells Department, Cell Sciences Research Center, Royan Institute, ACECR, Tehran, Iran
2. Oral and Maxillofacial Surgery Department, Taleghani University Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran
3. Clinical Science Department, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
4. Iranian Center for Dental Research, Shahid Beheshti University of Medical Sciences, Tehran, Iran

**** Corresponding Address: P.O. Box: 19395-4644, Stem Cells Department, Cell Sciences Research Center, Royan Institute, ACECR, Tehran, Iran***
Email: eslami@royaninstitute.org

Abstract

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Objective: Biphasic ceramics of hydroxyapatite and three calcium phosphate (HA/TCP) are increasingly being used as a bone substitute in regenerative surgery. To increase the bone forming capacities, HA/TCP Scaffolds could be enriched with osteogenic factor like mesenchymal stem cells (MSCs) which is the subject of present study.

Materials and Methods: Passaged-3 culture-expanded MSCs of canines bone marrow were suspended in a diluted collagen gel and loaded onto commercially-available HA/TCP ceramics. The cell-loaded scaffolds were then autologously implanted along with the control cell-free scaffolds in masseter muscles of the four mongrel dogs. Eight weeks later, the parts of their muscles including the implants were prepared for a light microscopy. To quantify the amount of bone formation, the slides of both studied groups were photographed and the percent area of the newly formed bone was calculated using Image-Pro Plust software.

Results: According to our observations, the implants were appeared to be encapsulated by fibrous tissue within the muscle. No cartilage tissues were observed in implantation site. Histological observation indicated that ectopic bone was formed in both MSCs-loaded scaffolds as well as the control cell-free implants. The percentage of newly formed bone for cell loaded HA/TCP scaffolds was %29.12±6.01 compared to %23.55±4.99 of the cell-free implants ($p<0.05$). Furthermore, lamellar mature bone was only observed in cells/scaffold groups.

Conclusion: Taken together, it seems that MSCs enhance bone formation capacity of HA/TCP. The formed bone following MSCs/scaffold composite implantation appeared to be histologically lymature lamellar bone.

Keywords: Canine Mesenchymal Stem Cells, HA/TCP Biphasic Ceramic, In Vivo Ectopic Bone Formation

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Introduction

The vast majority of defects in bone tissue could heal spontaneously with minimal therapy; but in some cases including large bone loss, it requires further treatment. In such situation, bone grafts and/or bone substitute were preferred (1). Autogenic bones graft, providing three essential components of osteoconductive, osteoinductive and osteogenic, are biocompatible and effects healing site. Although, the use of Autogenic bones graft is limited due to the lack of tissue supply, pain and morbidity which often develops at the donor site. (2-4).

Allogenic graft could be referred as an alternative option for repair of large bone defects. Such grafts providing only osteoconductive effect have limited

application since they present the risk of disease transmission and host rejection (5). The other option is the use of natural or synthetic bone substitute such as coralline hydroxyapatite, hydroxyapatite and β-tricalcium phosphate ceramics. The main advantages of these materials over bone grafts are their availability (2, 5).
Biphasic ceramics of hydroxyapatite and three calcium phosphate (HA/TCP) are increasingly being used as a bone substitute in orthopedic and maxillofacial regenerative surgery (6-8). According to some investigations, these scaffolds may possess osteoinductive property in addition to their osteoconductive effects, but these properties are not enough for complete regeneration of bone defects, particularly in the case of extensive tissue loss (9,10). To overcome this problem, one approach is to enrich them by osteogenic factor such as bone forming cells. In this regard, MSCs are preferred because they can easily be isolated and culture-expanded. Furthermore, they possess the ability to differentiate into multiple cellular phenotypes including the osteoblasts used as a promising cellular source for tissue engineering purpose (10-14).
In this study, bone marrow derived MSCs ,canine model, were loaded onto biphasic HA/TCP ceramics and then implanted in the master muscle of four dogs in order to compare the osteogenic activity in the MSCs loaded scaffolds group and control group. This study helps to understand in vivo bone formation capacity of commonly-used bone substitute (HA/TCP) when enriched by MSCs.

Materials and Methods

Animals and bone marrow aspirates

In this experiment, mongrel dogs with average weight of 15-25 kg were used. The study was approved by Institutional Animal Care and Use Committee of the Tehran, University of Medical Sciences, and it was according to the standards of Association for Assessment and Accreditation of Laboratory Animal Care. The animal was housed for 1 week to become acclimatized to housing and diet. Throughout the experiments, the animals were maintained individually in the cages, fed with nutripet (Behintash Company, Karaj, Iran) and monitored for general appearance, activity, exertion and weight. Under general anesthesia, bone marrow aspirate (about 10 ml) were drawn from the canine humerous, collected into 50 ml tube containing 7500 unit heparin and shipped on the ice to cell culture facility of Royan Institute

Cell culture

The marrow was mixed with three volumes of low-glucose DMEM(Dulbecco Modified Eagle Medium, Gibco , UL) containing 10% FBS(Fetal Cow Serum , Gibco ,UK) , 100 IU/ml penicillin G and 100 ug/ml streptomycin. The nucleated cell fraction of the marrow was enriched for MSCs, based on density separation on a 1.077 g/cm Lymphodex (Inno-Train, Sweden) cushion. For this purpose, 2×10^8 nucleated cells in 5ml of media were carefully layered over 20ml of Lymphodex and separated by a centrifuge at 400× g for 20min. Cells were collected at media-Lymphodex interface were washed and plated in 150-cm^2 flask at 5×10^4 cells /ml in 15 ml low-glucose DMEM containing 15% FBS, 100 IU/ml penicillin G and 100 IU/ml streptomycin. The cultures were incubated at 37°C and 5% CO_2. The non-adherent cells were removed by medium replacement on day 7. The cultures were fed twice a week. On days 17-2, the cells were detached by using 0.05% Trypsin and 0.53mM EDTA for 5 minutes and subcultured in 150-cm^2 culture flasks in 1:3 ratios. Two additional passages were performed to obtain adequate number of the cells and this was achieved upon passage 3.

Evaluation of the mesenchymal stem cell nature of the isolated cells

To identify the isolated cell as the MSCs, their differentiation potential into osteogenic, chondrogenic and adipogenic cell lineages were evaluated as detailed below.

Osteogenesis

Confluent passage-3 cells were provided with DMEM medium supplemented with 50µg/ml ascorbic2-phosphate (Sigma), 10 nM dexamethazone (Sigma) and 10 mM ß-glycerol phosphate (Sigma) for 3 weeks. At the end of this period, alizarin red staining was used to observe the deposition of mineralized matrix. For staining, the cultures were first fixed by methanol for 10 minutes and then subjected to alizarin red solution for 2 minutes.

Chondrogenesis

To induce the cartilage differentiation, micro mass culture system was used. For this purpose, 2.5×10^5 passage-3 cells were pelleted under 1200g for 5 minute and cultured in DMEM medium supplemented by 10ng/ml transforming growth factor-ß3 (TGF-ß3; Sigma), 10ng/ml bone morphogenetic protein-6 (BMP-6; Sigma), 50 mg/ml insulin transferin selenium+ premix (Sigma), 1.25 mg bovine serum albumin (BSA; Sigma) and 1% fetal bovine serum (FBS; Sigma). After 3 weeks; the pellets were sectioned at 5 µm, stained by toluidine blue for 30 second at the room temperature and observed by a light microscope.

Adipogenesis

To induce adipose differentiation, passaged-3 confluent culture was treated with the medium containing 100nM dexamethazone (Sigma) and 50 mg/ml indomethasine (Sigma). After 3 weeks, the cells were fixed with 4% formalin at the room temperature, washed with 70% ethanol and stained by oil red solution in 99% isopropanol for 15 minute.

RNA extraction and RT-PCR analysis of gene expression

The differentiation ability of the cells and some specific gene expression were also studied by RT-PCR analysis. For this purpose, the total RNA collected from the cells differentiated into the bone, cartilage and adipose cells as detailed above, using RNX-PlusTM solution (CinnaGen Inc., Tehran, Iran). Before the reverse transcription, the RNA samples were digested with DNase I (Fermentas) to remove the contaminating genomic DNA. Standard reverse-transcription reaction was performed with 2µg total RNA using Random Hexamer as a primer and Revert Aid TM H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacture's instructions. Subsequent PCR was as following: 2.5 µl cDNA, 1X PCR buffer (AMS), 200 µM dNTPs, 0.2 µM of each primer pair and 1 unit/25 µl reaction Taq DNA polymerase (Fermentas). The primers indicated in Table 1 were used to detect differentiation. Each PCR was performed in triplicate. The products were analyzed on 2% agarose gel and visualized by ethidium bromide staining.

Implant preparation

In this study, HA/TCP implants in the form of 3-mm cubes (Kasios, France) were used. The day before transplantation, scaffold was loaded with the cells obtained from third subculture. To increase the loading efficiency, 5×105 MSCs cells were suspended in 0.5 ml DMEM medium-diluted collagen gel (Vitrogen, Chohesion, Sweden) and placed on the top surfaces of implants cubes. The same amount of collagen gel was used to prepare the cell-free scaffold for the control group. To observe the cells loaded within scaffolds pore system, some cell loaded implants were decalcified, formalin-fixed, dehydrated and embedded in paraffin blocks. Five micrometer sections were then prepared, stained with H & E and observed by a light microscope.

Surgical procedure

Four adult mongrel dogs with healthy teeth, weighing between 20-30 kg, were used in this study. The dogs were premeditated with Xylazine- HCl (1 mg/kg) (Xylazine 2%, Alfasan, Woerden-Holland) intramuscularly and atropine sulphate (0.05 mg/kg) (Atropin 0.5, Daroupakhsh Pharmaceutical Mfg, Co, Tehran, Iran), subcutaneously. This was followed by general anesthesia with sodium thiopental (10 mg/kg) (Nesdonal, Specia, France) intravenously and oroendotracheal intubation. After induction of general anesthesia, infiltration anesthesia was applied to the submandibular body area. Submandibular incision was made in the bilateral mandibular angle area, and layered dissection was performed through the mandibular bone. Layered dissection was performed until investing fascia of the masseter muscle reached. Blunt dissection with curved hemostat performed to create tunnel pouch measured 5×5 millimeter. In the right side of the mandible 4 granules of HA/TCP loaded with cMSCs were embedded with microforceps; and in the other side, the same amount of control cell free scaffolds was embedded. The pouch was closed in the layered fashion with resorbable sutures (Vhicril, Ethicon).

Qualitative and quantitative histological study

Eight weeks after insertion of the implant, the implant site of the muscle were removed and placed in 10% formalin for 10 days and decalcified in formic acid for 24 days. The specimens were washed with tap water, dehydrated with ascending concentrations of ethyl alcohol, cleared in xylene, infiltrated with paraffin and processed for histological evaluation. Decalcified coronal 5 µm serial sections which incorporated total implant area were prepared and stained using haematoxylin & eosin.

The stained sections were examined under a light microscope in terms of the bone formation, the presence of scaffold piece, and the presence of inflammation cells. Furthermore, to quantify the amount of bone formation, all slides were photographed at 6.5 magnification using stereomicroscope equipped with digital camera (Nikon E8400, Japan). The percent area of the bone formation was then calculated for either scaffold/cell or cell free scaffold implants using Image-Pro Plust software (Media Cybernetic, Silver Springs, MD, USA).

Statistical analysis

All the data are presented as means and standard deviation. The data were subjected to statistical analysis using one-way ANOVA and Post-Hoc Tukey. Differences at $p<0.05$ were considered significant. Calculation was performed using the SPSS statistical package (SPSS 11, SPSS Inc., Chicago, IL, USA).

Results

Cell culture

Two kinds of cells were observed in the primary cultures of the canine's bone marrow: fibroblastic and small round cells (Fig 1A). The number of the round cells appeared to be reduced by performing subcultures during which the fibroblastic MSCs became purified. Upon passage three, the number of the cells seemed to be in sufficient number to conduct the rest of the experiment (Fig 1B).

Differentiation

According to alizarin red staining results, fibroblastic cells isolated in this study colored red (Fig 2A) indicating that mineralized matrix was produced. Upon toloidine blue staining, the sections of the cells differentiated in the micro mass culture system stained purple (Fig 2C) because of their glycoseaminoglycan-rich matrix.

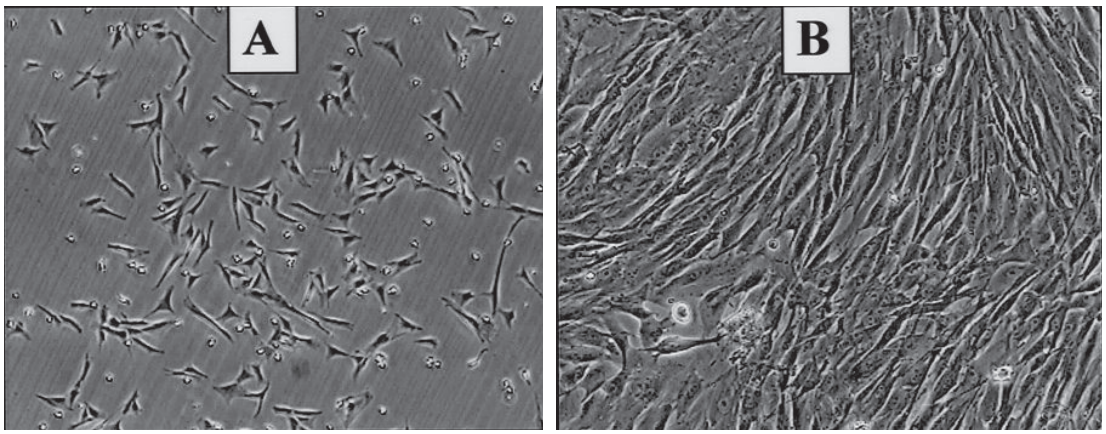

Fig 1: Canine MSCs isolation and expansion A) Primary culture, bar: 150 µm B) Passage 3, bar: 100 µm.

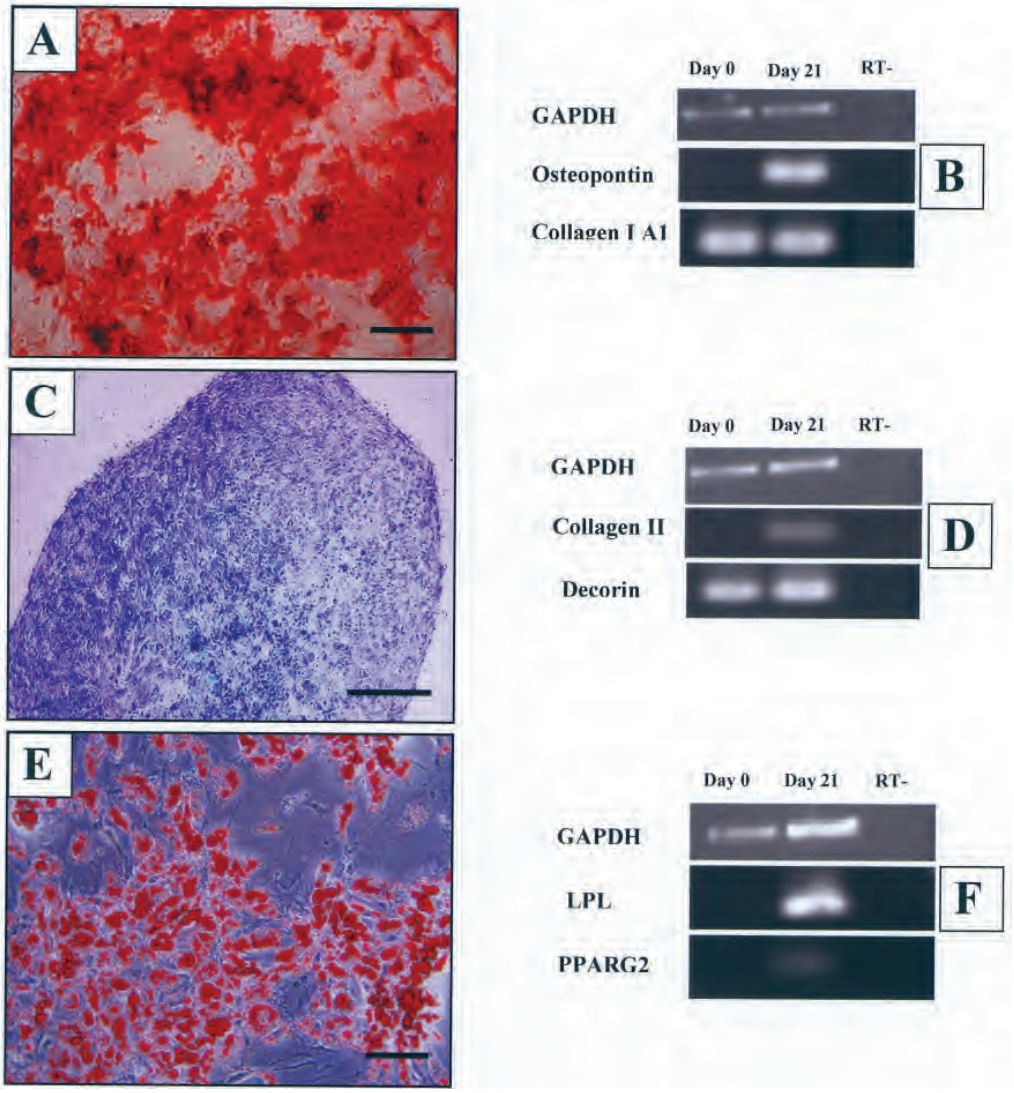

Fig 2: In vitro differentiation of the culture-expanded cMSCs. A) Alizarin red staining of osteogenic culture, bar: 100 µm. B) Gene expression in osteogenic culture, C) Toluidin blue staining of cartilage differentiation, bar: 100 µm D) Chondrocyte specific genes including collagen II expressed in chondrogenic culture. E) Oil red staining of adipogenic culture, F) Adipocyte specific genes expressed in differentiated culture, bar: 100 µm.

The lipid droplets appeared in the cells subjected to adipogenic differentiation were oil red positive (Fig 2E); a specific dye for adipocyte detection. According to RT-PCR analysis, there were indications that the specific genes of osteocytic (Fig 2B), chondrocytic (Fig 2D) and adipocytic (Fig 2F) cell lineages were expressed in the differentiated cells.

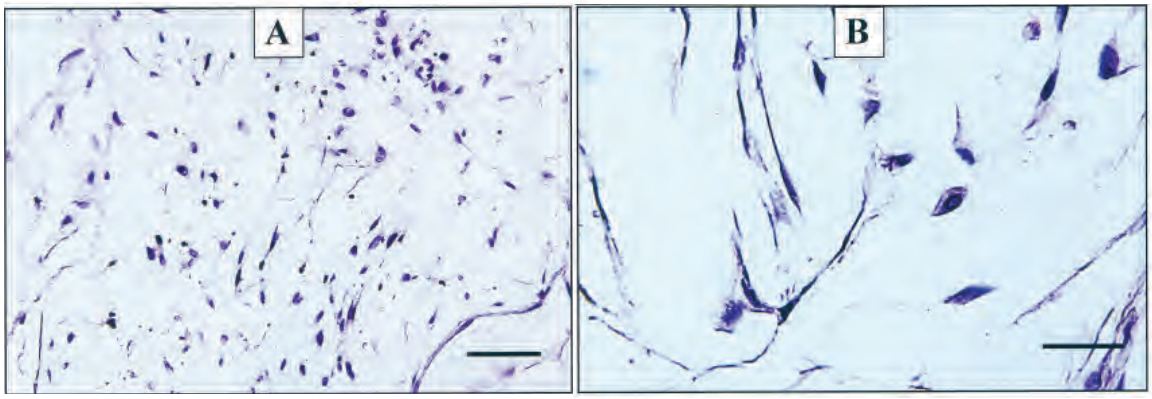

Fig 3: Decalcified scaffolds contained the loaded MSCs A) bar: 100 µm and B) bar: 30 µm.

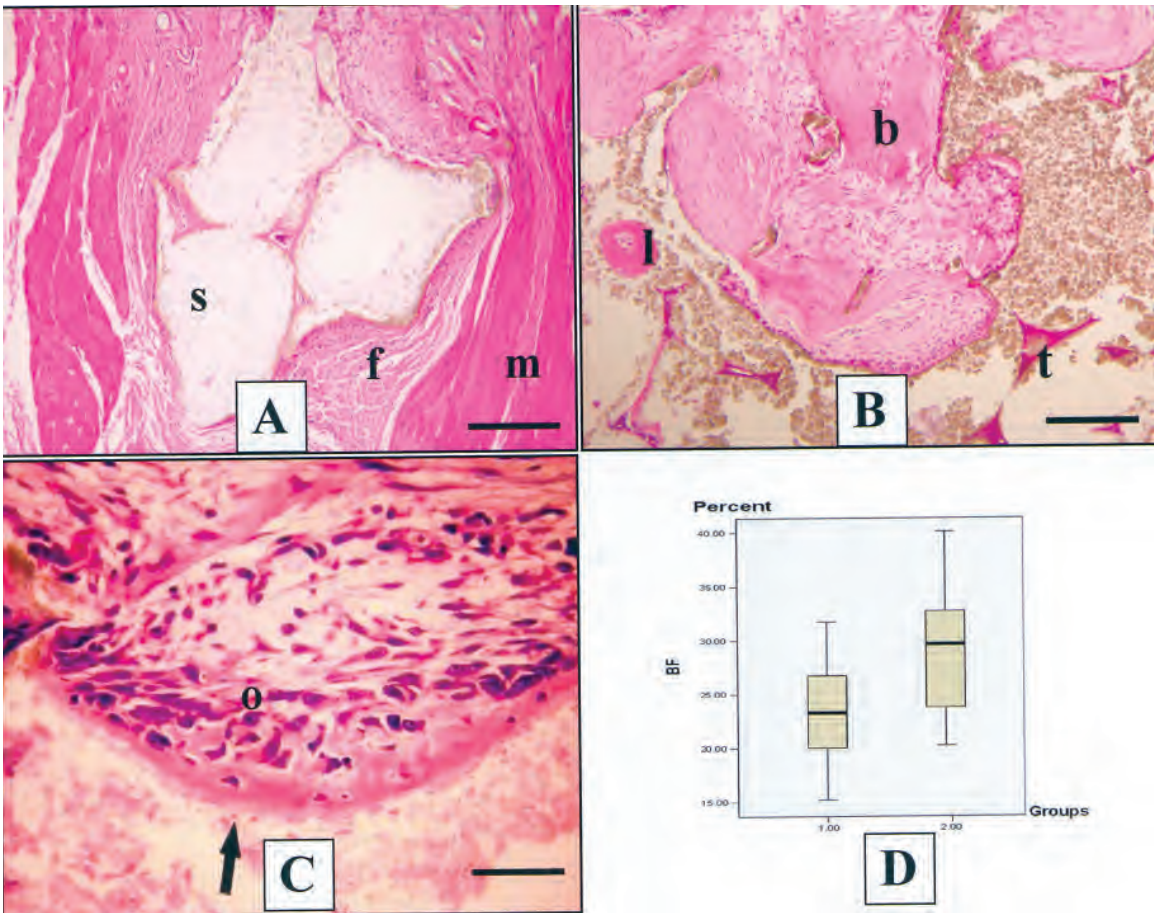

Fig 4: Representative histomicrograph of the bone formation within the muscle tissue. A) Scaffolds encapsulated by fibrous tissue (f) in both groups, s: scaffolds, m: muscle fibers, bar: 200 µm, H&E staining. B) Only in test group, the laminar bone was observed (I), b: newly formed bone, t: bone trabucula, bar: 200 µm, H&E staining. C) Bone formation site included osteoblast rim (o) and newly formed osteoid (arrow) bar: 50 µm, H&E staining. D) The percentages of the newly formed bone were significantly high in tested group compared to control group, BF: bone formation, 1.00: control group, 2.00: tested group.

Cell loading into scaffold

Observing the sections prepared from the cell-loaded scaffolds indicated that the cells used to seed the implants remained within the scaffold and well occupied its internal spaces (Fig 3A, B).

Evaluation of in vivo bone formation

Qualitative data

According to our observations, the implants were appeared to be encapsulated by fibrous tissue within the muscle (Fig 4A). Inflammation was very mild in all specimens. In some area scaffolds particles were being phagocytized by macrophages. A few giant cells were also observed in the sections. In some area, the penetrations of the vessels into the scaffolds particles were evident. In almost all specimens, osteogenesis was clearly visible.

In contrast to cell free control, in the scaffold/cells group, the bone being formed in implantation site appeared to be structurally mature lamellar bone (Fig 4B). No cartilage tissues were observed in implantation site. The bone trabecula formed in implantation site contained the osteoblastic rim and recent osteoid formation(Fig 4C). The osteocytes were also visible within the lacunae- like cavity of the bone trabeculae.

Quantitative data

The percentage of newly formed bone in implantation site of the cell loaded HA/TCP scaffolds was %29.12±6.01 compared to %23.55±4.99 of the cell free implants (Fig 4D). This difference was statistically significant ($p<0.5$).

Discussion

In present study, biphasic calcium phosphate scaffolds loaded with canine MSCs were autologuosley implanted in masseter muscle tissue along with the control cell free scaffolds and subsequent ectopic bone formation was studied and compared, using qualitative and quantitative analysis of histological section prepared from the implant sites. Histological observation indicated that ectopic bone was formed in both MSCs loaded and the control cell-free implants. The main differences among them were in terms of the amount as well as the kind (imature or mature) of bone formed in implantation site. Our quantitative results suggested that in cell-loaded scaffold group the amount of bone formation was significantly higher than that of the control cell free implants. Furthermore, in contrast to the control group, in MSCs group, lamellar bone was observed in some area.

In vivo bone formation, the result of a complex process could be influenced by (i) the biomaterial, itself, (ii) the host dependent parameters and (iii) the donor-cells including their osteogenic cell commitment. Since all parameters in our experiment were the same in both groups except the present of MSCs, augmented bone formation in the Scaffolds/MSCs group could be considered as a consequence of MSCs activity. The Implantation of the culture-expanded autologous MSCs offers the advantage of directly delivering to the cellular machinery being responsible for synthesizing new bone and circumvents the otherwise slow steps leading to natural or enhanced bone repair. By incorporating living cells with specifically designed matrices, the shortcomings of osteoconductive factors alone to affect permanent bone repair may be overcome.

Up to now, a few studies have also considered the in vivo ectopic bone formation of cell loaded HA/TCP. Cooper and associates reported that human MSCs loaded onto HA/TCP could generate embryonic bone after implantation in subcutaneous tissue of nude rat (15). In other study, De kok and co-workers you have to mention the year in here!!!! who implanted cell loaded HA/TCP scaffolds in canine premolar tooth reported that the embryonic bone was formed (16). These results and our finding indicated a kind of lamellar bone formation in the implantation site. These differences could be explainable with considering the fact that the kind of animal model as well as the implantation site used was different from the mentioned studies.

Regarding the treatment of bone defects, there are three strategies in the field of orthopedic and maxillofacial surgery. One strategy would be the local recruitment of osteogenic cells by BMPs. Presence of BMPs specially BMP-2 and BMP-6 in defect site creates an osteogenic microenvironment that allows multipotent cell progenitors to migrate to the area of injury, proliferate and differentiate toward the osteogenic lineages. A main concern related to the use of BMPs is to supply large dose of non-physiologic BMPs for producing their beneficial effects (17). Systemic injection of postnatal multipotent cells would be an alternative way to treat bone diseases. Using this strategy, Horwitz et al reported an improvement of the clinical condition in three children with severe osteogenesis imperfect after injection i.v. with allogenic bone marrow-derived MSCs (18-19). Nevertheless, it is hardly conceivable that a similar approach could be of any benefit for the treatment of large bone deficits. For such defects, implantation of postnatal multipotent cells in association with ceramic scaffolds is considered to be more appropriate strategy (20-21). The present investigation is designed according to this strategy. Experiments like this can help to understand the potential of scaffold/cell combination in producing bone tissue in vivo.

One limitation of MSCs study was due to unavailability of distinct specific markers introduced to them because of this, their identifications among the others and their isolations were difficult task. In the lack of specific marker, it was proposed that the golden standard to identify the MSCs is to differentiate them into two or more cell lineages (17). In present study,

evaluation of the isolated cells indicated that they were able to produce differentiated progenies including osteoblastic, chondrocytic and adipocytic lineages, therefore; their mesenchymal stem cell nature was confirmed.

In present study, when the cells suspended in medium alone loaded into scaffold, no cells could adhere on internal surfaces of the scaffolds because they rapidly passed through the scaffold's pores and left from the other side. This occurred probably because the scaffolds were highly porous (70%-75%) with large pore size of 150-300 µm. To overcome this problem, the medium, used for cell loading, was rendered a little more viscous by adding a few µl of collagen I gel, and the cell suspended in this mixture was placed on top surface of the scaffold as a drop. This procedure provides the cell with a chance of slow penetration and enough time of interacting with internal surfaces of the scaffolds.

Conclusion

Taken together, it seems that the amount of ectopic bone formation enhances when HA/TCP combined with MSCs. Fourthermore, MSCs directed osteogenisis could be resulted in the formation of mature lamellar bone in transplantation spot.

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