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Enhancing Ectopic Bone Formation in Canine Masseter Muscle by Loading Mesenchymal Stem Cells onto Natural Bovine Bone Minerals

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Abstract

Objectives- To assess the ectopic bone formation in canine masseter muscle following the implantation of the natural bovine bone minerals (NBM) loaded with canine mesenchymal stem cells (MSCs).
Design- Experimental study
Animals- four mongrel dogs
Procedures- Tripotent MSCs isolated from the canine bone marrow were loaded onto the NBM sponges and allowed to adhere. The cell-loaded scaffolds were then implanted in parallel with cell-free control scaffolds in masseter muscles of four mongrel dogs. Eight weeks after, the animals were sacrificed and the ectopic bone formation in implantation site was studied using the sections prepared from the parts of the muscle containing the implants. Furthermore, the amount of bone formation in two studied groups was quantified using Image-Pro Plust software.
Results- The implants from the both groups were appeared to be encapsulated by fibrous tissue in implantation site which included some trabecular bone containing osteocyte and osteoblast. There were no indications of inflammation and foreign body reaction, nor were there any indications of cartilage tissue formation.In contrast to control, in MSCs group, lamellar bone

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was observed in some area. More importantly, in cell loaded scaffolds more amount of bone was formed compared to that of control cell free scaffolds ($P<0.05$).
Conclusion and Clinical Relevance- Taken together it seems that in vivo bone forming capacity of the NBM sponges would be improved by loading it with MSCs.
Keywords- Mesenchymal Stem Cells, Bovine Bone Minerals, Canine.

Introduction

Large bone defects represent major clinical problems in the practice of reconstructive orthopedics and craniofacial surgery. As current treatment for these applications, such as autogenous or allogenous bone grafting have limitations, new approaches for bone tissue repair are essential [1-5]. In this regards bone tissue engineering approach using mesenchymal stem cells and appropriate scaffold has been gained considerable attention [6,7].
Mesenchymal stem cells are multipotential cells capable of differentiating into osteoblasts, chondrocytes, adipocytes, tenocytes, and myoblasts [8-10]. From a small bone marrow aspirate (~10 to 20 mL), mesenchymal stem cells can be isolated and expanded in culture into a large number because of their extensive proliferative capacity [11,12].
Calcium and phosphate ceramics are being increasingly used as bone substitutes in orthopaedic, oral and maxillo-facial surgery [13-15]. Yoshikawa et al [16] have reported that hydroxyapatite (HA) loaded with mesenchymal stem cells (MSCs) has osteogenic potential comparable with autogenous particulate cancellous bone and marrow (PCBM). Furthermore, Yamada et al. have confirmed similar osteogenic potential in ß-TCP (tricalcium phosphate), which is a biodegradable material [17].
BioOss®, a natural bovine bone mineral is being largely used in reconstructive craniofacial surgery as a bone fill. This scaffold is indeed a bovine deproteinized bone sterilized by gamma radiation and possesses osteoconductive property. A few in vitro studies have confirmed that this scaffold could support fibroblastic and mesenchymal stem cell culture, but little is known regarding their in vivo bone formation capacity when being loaded with bone forming cells [18-21].
In present study, canine bone-marrow derived mesenchymal stem cells were loaded onto BioOss sponges and engrafted autologously in the canine masseter muscle to evaluate the ectopic bone formation of the cell/scaffold construct compared to that of cell-free materials. Study like this could help to understand in vivo bone formation capacity of commonly-used bone substitute (BioOss) when being enriched by MSCs.

Materials and Methods

Mesenchymal Stem Cell Isolation and Cultivation
Mongrel dogs with average weight of 15-25 kg were used for this experiment. This study was performed in accordance with the regulations and approval of Institutional Animal Care and Use Committee of the Tehran University of Medical Sciences and conformed to 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 was 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. In the cell culture lab, canine MSCs were isolated according to the method by Kadiyala et al[18] with some modification. In brief, the nucleated cell fraction of the marrow was enriched by gradient centrifugation and cultured in 150-cm^2 flask at 5×10^4 cells /ml in 15 ml low-glucose DMEM (Dulbecco Modified Eagle Medium, Gibco, UL) containing 15% FCS, 100 U/ml penicillin G and 100 U/ml streptomycin. The cells were incubated at 37 °C in a humidified 5% CO2 atmosphere and on day 7, the non-adherent cells were removed along with the culture media. The cultures were feed twice a week, passaged on days 17-21 by lifting the cells with 0.05% Trypsin 0.53 mM EDTA exposure for 5 minutes and split in a 1:3 ratio into new 150-cm^2 culture flasks. Additional passages were performed to obtain adequate number of the cells and this was achieved upon passage 3.

Differentiation Potential

To evaluate the mesenchymal stem cell nature, the isolated cells were differentiated into osteogenic, chondrogenic and adipogenic cell lineages.

To induce osteogenic differentiation**,** confluenced passaged-3 cells were cultured in the DMEM medium supplemented with 50 mg/ml ascorbic2- phosphate (Sigma, USA), 10 nM dexamethazone(Sigma, USA) and 10 mM b gicerole phosphate (Sigma, USA) for 3 weeks. At the end of this period, alizarin red staining was used to observe the matrix mineralization. For staining, the cultures were first fixed by methanol for 10 minutes and then subjected to alizarine red solution for 2 minutes.

To induce the cartilage differentiation, micro mass culture system was used. For this purpose, 2.5×10^5 passaged-3 cells were pelleted under 1200 g for 5 minute and cultured in a DMEM medium supplemented by 10 ng/ml transforming growth factor-b3, 10 ng/ml bone morphogenetic protein-6, 50mg/ml insulin transferin selenium+ premix and 1.25 mg bovine serum albumin and 1% fetal bovine serum. Three weeks later, the pellets were subjected to the following: fixing in 10% formalin; dehydrating in an ascending ethanol; clearing in xylene; embedding in paraffin wax and sectioning in 5µ by microtome. The sections were then stained by toluidin blue for 30 second at room temperature.

For adipogenesis**,** DMEM medium containing 100 nM dexamethazone (Sigma, USA) and 50 mg/ml indomethasine (Sigma, USA) was used to induce the differentiation in the confluenced culture of the cells. Three weeks later, the culture was fixed with 4% formalin at room temperature, washed by 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 was also studied by RT-PCR analysis of some specific gene expression. For this purpose, total RNA was collected from the cells having been induced to differentiate into bone as detailed above, using RNX-Plus^{TM} solution (CinnaGen Inc., Tehran, Iran). Before reverse transcription, the RNA samples were digested with DNase I (Fermentas) to remove contaminating genomic DNA. Standard reverse-transcription reaction was performed with 5 µg total RNA using Oligo (dT) $_{18}$ as a primer and RevertAid ^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacture's instructions. Subsequent PCR was as follows: 2.5 µl cDNA, 1X PCR buffer (AMS), 200 µM dNTPs, 0.5 µM of each primer pair and 1 unit/25 µl reaction Taq DNA polymerase (Fermentas). The primers indicated in Table 1 were utilized to detect osteoblastic differentiation. Each PCR was performed in triplicate and

under linear conditions. The products were analyzed on 2% agarose gel and visualized by ethidium bromide staining.

Table 1: The primers used in RT-PCR analysis

Genes Code	Primer sequences(5´-3´)	Annealing temperature (°C)	Length bp	Gene bank code
Col IA1	F: 5' tca cct acc act gca aga ac 3' R: 5' agt tta cag gaa gca gac agg 3'	62	302	NM_001003090
COL IIA1	F : 5' caa gaa cag cat tgc cta cc 3' R : 5' agt tag ttt cct gcc tct gc 3'	57	550	NM_001006951
COL IA2	F : 5' tca cct acc act gca aga ac 3' R2 : 5' tga aac aga ctg ggc caa cg 3'	60	294	NM_001003187
LPL	F : 5' gtg aac atg tgt ggg tat ctg R : 5' cta ggg cct tta ctg act gga	65	370	XM_534584.2
Aggrecan	F : 5' aca gga ttg aag tca gtg gag 3' R : 5' gtt gac aaa ctc ctg ttc ctc 3'	63	527	U65989
GAPDH	F : 5' cca cgg caa att cca cgg cac ag 3' R : 5' ggg gtc cct ccg atg cct gct tc 3'	57	652	NM_001003142
Decorin	F : 5' aac cag atg atc gtc gta gag 3' R : 5'gat gag gag tgt tgg cta gag 3'	60	292	NM_001003228
Osteopontin	F: 5'acg atg tag atg aag atg atg g 3' R: 5'gct ttg act taa ttg gct gac 3'	57	548	XM_535649
PPARG	F : 5' gaa tta gat gac agc gac ttg g 3' R : 5' cag tga att tgg act tct ctg c 3'	59	324	NM_001024632
PPARG 2	F : 5' atc cct ctt cca tgc tgt tat g 3' R : 5' ata gtg tgg agt gga aat gct g 3'	62	253	AJ972913

Implant Preparation

One day before transplantation, BioOss (Geistlich, Osteohealth Biomaterials, Bern, Switzerland) implants in 1-2-mm granules were loaded by the cells obtained from third subculture. For this purpose, 250 µl of collagen gel (Vitrogel®) was mixed with 250 µl of DMEM and the pH was adjusted to 7.4 by 1N NaOH dropwise. 5×10^5 MSCs were then suspended homogenously in 500 µl of diluted collagen gel and placed on the top surfaces of the implants granules. To observe whether or not the cells being loaded traped in scaffold pore system and adhered to its surfaces , samples were decalcified, fixed over night in 10% formaldehyde in PBS buffer, washed with tap water, dehydrated with an ascending row of ethanol (Merck, Darmstadt, Germany) and finally

embedded in paraffin wax (Leica, Bensheim, Germany). Five micrometer sections were then cut, stained with H & E and observed by light microscope.

In-vivo Bone Differentiation

Four adult mongrel dogs with healthy teeth, weighing between 20-30 kg, were used in this study. The dogs were premedicated 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 anaesthesia with sodium thiopental (10 mg/kg) (Nesdonal, Specia, France) intravenously and oroendotracheal intubation. After induction of general anaesthesia, infiltration anaesthesia 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 creat tunnel pouch measured 5×5 millimeter. In the right side of the mandible 4 granules of BioOss loaded with cMSCs were embedded with microforceps and in the other side the same amount of control cell free scaffolds were embedded. The pouch was closed in the layered fashion with resorbable sutures (Vhicril, Ethicon). Each dog was sacrificed 8 weeks after insertion of the implant with an overdose of sodium thiopental and subsequent perfusion through the carotid arteries with a fixative consisting of a mixture of glutaraldehyde (5%) and formaldehyde (4%) buffered to pH 7.2 .The implant site of the muscle were removed and placed in 10% formalin for an additional 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 histologic evaluation. Decalcified coronal 5 μm serial sections which incorporated total implant area were prepared and stained using H & E.

The stained sections were examined under light microscope in terms of the bone formation, the presence of scaffold piece, the presence of inflammation cells. Furthermore, to quantify the amount of bone formation, slides were photographed at 6.5 magnification using stereomicroscope equipped with digital camera (Nikon E8400, Japan). The percent area of 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 were performed using the SPSS statistical package (SPSS 11, SPSS Inc., Chicago, IL, USA)

Results

Cell Culture

The primary cultures of the canine's bone marrow mononuclear cells contained some fibroblastic cells as well as a few small round cells (Fig. 1A). The number of the latter was reduced by performing subcultures during which the fibroblastic MSCs were purified and expanded. (Fig.1B)

Differentiation

Fibroblastic cells isolated in this study were readily differentiated into bone, cartilage and fat cells as confirmed by alizarin red staining for mineralized matrix (Fig. 1C), Oil red staining for lipid droplets of adipocytic cells(Fig. 1E) and toluidine blue for methachromatic matrix of cartilage(Fig. 1G). According to RT-PCR analysis there were indications that the specific gene markers of above-mentioned cell lineages were strongly expressed in differentiated cells (Fig. 1D, F and H).

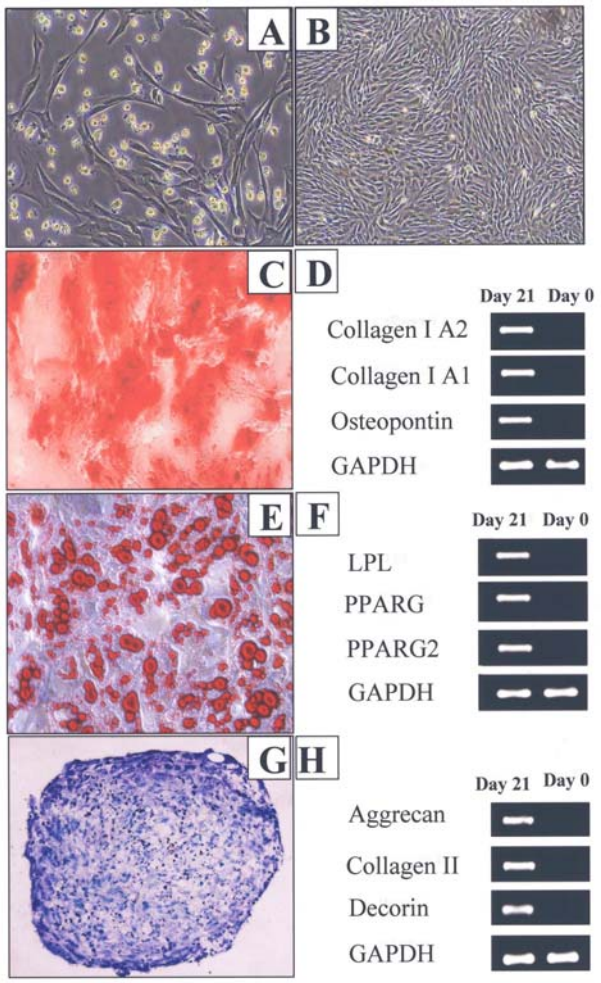

Figure 1: Canine MSCs isolation, expansion and differentitian. A) Primary culture, B) Passage 3 , C) Alizarin red staining of osteogenic culture, D) Bone gene expression of the differentiated cells, E) Oil red staining of adipogenic culture, F) Adipocyte specific genes expressed in differentiated culture, G) Toluidin blue staining of cartilage differentiation, H) Chondrocyte specific genes expressed in differentiating cultures.

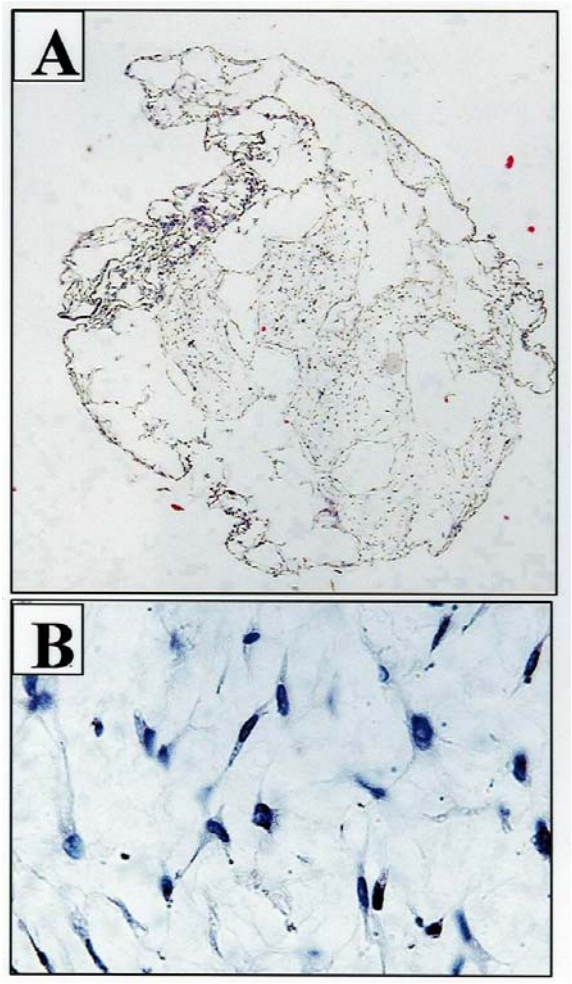

Figure 2: Decalcified scaffolds contained the loaded MSCs A: magnification 10× and B) magnification 200×

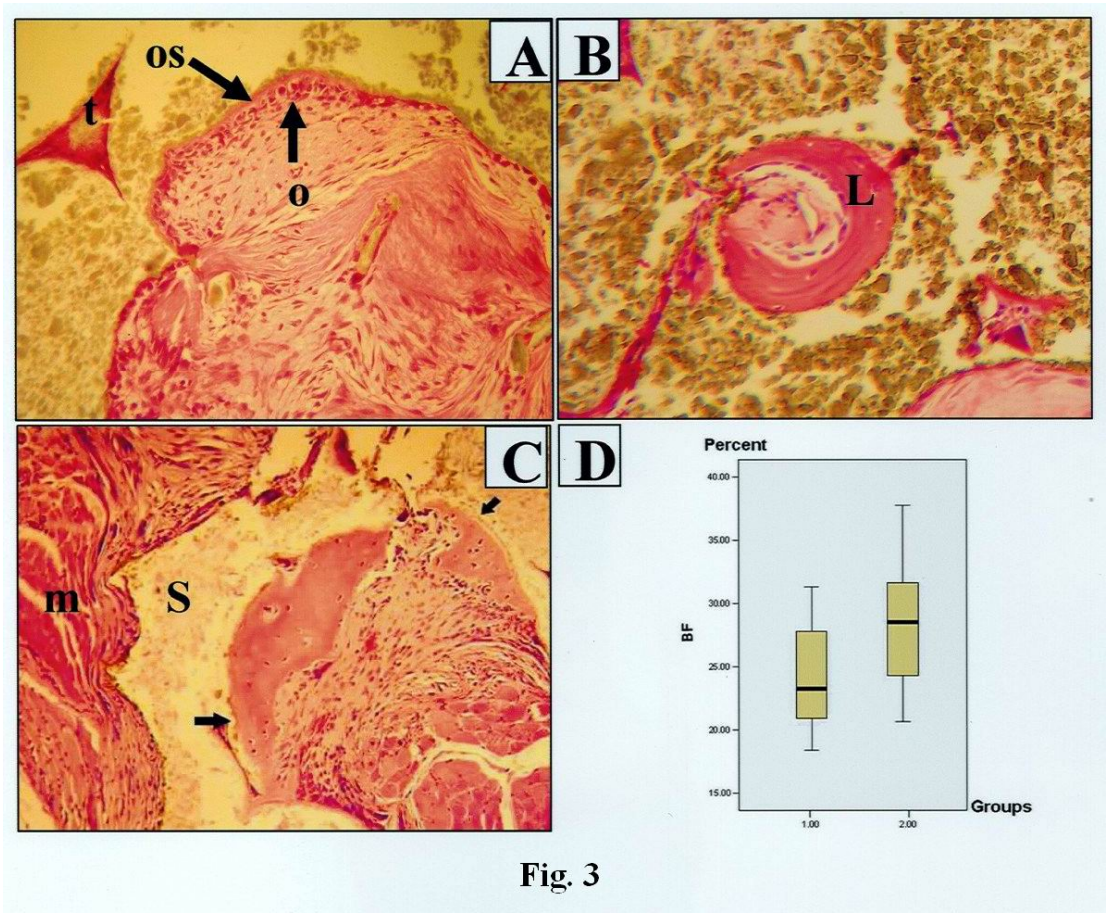

Figure 3: Representative histomicrograph of the bone formation within the muscle tissue. A) Bone trabecula (t) was visible in implantation site in both groups. In some area, osteoblastic rim(o) and the newly formed osteoid was evident(os), Magnification 100×, H&E staining. B) Only in test group the laminar bone(L) was observed, Magnification 200×, H&E staining. C) The trabecula (Arrow) included many osteocytes and was lined with many osteoblasts. m: muscle fiber, s: scaffold, Magnification 100×, H&E staining D) The percentages of the newly formed bone were significantly high in test compared to control group, BF: Bone formation , 1.00: Cell free scaffold, 2.00: Cell/scaffold composite.

Decalcification

According to the sections prepared from cell-loaded scaffolds, the spaces within BioOss granules appeared to be occupied by canine mesenchymal stem cells. The cells were observed to be morphologically spindle-shape and adherent to scaffold internal surfaces (Fig. 2A and B).

Histologic Study

A histological examination of the repair site revealed that the implants were encapsulated in a fibrous tissue, and that there was trabecular bone as well as an amorphous calcified matrix in all the samples (Fig. 3A). The trabeculae included many osteocytes and were regularly lined with many osteoblasts, indicating bone-forming activity (Fig. 3B). In some area of test group, the bone had a laminar pattern similar to normal bone (Fig. 3C). In this bone formation process, cartilage

formation was not found at any time, and thus the process was the so-called intramembranous bone formation. There was a generalized absence of inflammation or foreign-body reaction in the sections.
The percentage of newly formed bone was *28.18%± 5.20* in the BioOss/MSCs implants while in the control cell free matrices it was *24.16%± 4.22* (Fig. 3D). This demonstrated that there were differences in the amount of new bone formed in response to the MSCs and it was statistically significant ($p<0.05$).

Discussion

In present study bone marrow-derived canine MSCs were loaded onto sponges of natural bovine bone minerals (BioOss) and implanted autologously in masseter muscle for a period of 8 weeks. Histological observation indicated that ectopic bone was formed in both MSCs loaded-scaffolds as well as the control cell-free implants. The main differences between two studied groups were in terms of the amount of bone being formed in implantation site as well as its histologic structure. Our quantitative results suggested that in cell-loaded scaffolds the amount of bone formation was significantly higher than of the control cell free implants. Furthermore, in contrast to control, in MSCs group, lamellar bone was observed in some area.
Implantation of culture-expanded autologous MSCs offers the advantage of directly delivering the cellular machinery responsible for synthesizing new bone and circumventing 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. A distinct advantage of using MSCs is that they are adult stem cells, isolated from donors capable of informed consent.
The observations made by Cooper et al (2004) indicated that BioOss loaded with human mesenchymal stem cells formed no bone when implanted in subcutaneous tissue of nude rats[20]. Our results stand in opposition with the report of Cooper et al in that we observed indications of lamellar bone formation in implant site. Such discrepancy in results could be attributable to differences in the kind of animal model (rat versus dog), implantation site (subcutaneous versus muscle tissue) and the period of implant maintenance (6 weeks versus 8 weeks) in each study.
The findings by Mauney et al (2005) who implanted MSCs loaded BioOss subcutaneously in nude rats revealed that the histologic kind of the bone being formed in implantation site was embryonic [21]. According to our results the bone being formed in implantation site seemed to be structurally mature bone. Again these differences could be explainable with considering the differences in each study setup including the kind of animal model and implantation site.
One limitation of MSCs study is that no distinct specific markers were introduced for them and for this reason, their identification among the others; hence their isolation would be a 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 [22]. 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 were loaded onto 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 that was 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. This procedure provides the cell with a chance of slow penetration and enough time of interacting with internal surfaces of the scaffolds.
Taken together it seems that in vivo bone forming capacity of the natural bovine bone mineral sponges would be improved by loading it with MSCs.

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چکیده

تقویت استخوان سازی اکتوپیک در داخل عضله ماضغه سگ از طریق بارگیری سلول های بنیادی مزانشیمی در داربست متشکل از مواد معدنی طبیعی گاو

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هدف- بررسی تشکیل استخوان اکتوپیک در داخل عضله ماضغه سگ به دنبال پیوند داربست های متشکل از مواد معدنی طبیعی بار گیری شده با سلول های بنیادی مزانشیمی..

طرح مطالعه- مطالعه تجربی

حیوانات- چهار راس سگ

روش کار- سلول های بنیادی مزانشیمی مشتق از مغز استخوان سگ، با توان تمایز به سه رده سلولی، به داخل اسفنج های متشکل از مواد معدنی گاوی بارگذاری شد و به مدت چند روز کشت گردید تا سلول ها به سطوح آن بچسبند. سپس اسفنج های حاوی سلول بطور اتولوگ، به داخل بافت عضله ماضغه سگ کاشته شد. اسفنج های بدون سلول نیز به عنوان گروه کنترل در عضله ماضغه طرف مقابل پیوند شد.. هشت هفته بعد، سگ ها قربانی شدند و تشکیل استخوان اکتوپیک با تهیه برش های بافتی از عضله حاوی ایمپلنت، بررسی شد. همچنین در مطالعه حاضر، میزان استخوان تشکیل شده در دو گروه اسفنج حاوی سلول و اسفنج بدون سلول با استفاده از نرم افزار Image-Pro Plust بررسی گردید.

نتایج- بر اساس مشاهدات ما، ایمپلنت ها از اطراف توسط بافت فیبروزی احاطه شده بود. ترابکول های استخوانی در هر دو گروه قابل مشاهده بود. این ترابکول ها، حاوی استئوسیت و استئوبلاست بود. در هر دو گروه ، هیچ نشانی از تشکیل بافت غضروفی ، التهاب و واکنش جسم خارجی وجود نداشت. بر خلاف گروه کنترل، در گروه اسکافولد حاوی سلول، در مناطقی استخوان لاملار بالغ مشاهده شد. نکته مهمتر این بود که در گروه آزمایش میزان استخوان ساخته شده، بطور معنی داری بیش از گروه کنترل بود.

نتیجه گیری و کاربرد بالینی- روی هم رفته به نظر می رسد که ظرفیت استخوان سازی مواد معدنی طبیعی گاو، با بارگیری سلول بنیادی مزانشیمی بهبود می بخشد.

کلید واژگان- سلول های بنیادی مزانشیمی، مواد معدنی استخوان، گاو، سگ.