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In Vivo Osteoinductive Effect and *In Vitro* Isolation and Cultivation Bone Marrow Mesenchymal Stem Cells

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A B S T R A C T

Bone marrow contains cell type termed Mesenchymal Stem Cells (MSC), first recognized in bone marrow by a German pathologist, Julius Cohnheim in 1867. That MSCs have potential to differentiate in vitro in to the various cells lines as osteoblast, chondroblast, myoblast and adipoblast cells lines. Aims of our study were to show in vivo capacity of bone marrow MSC to produce bone in surgically created non critical size mandible defects New Zeeland Rabbits, and then in second part of study to isolate in vitro *MSC from bone marrow, as potential cell transplantation model in bone regeneration.* In vivo *study showed new bone detected on 3D CT reconstruction day 30, on all 3 animals non critical size defects, treated with bone marrow MSC exposed to the human Bone Morphogenetic Protein 7 (rhBMP-7). Average values of bone mineral density (BMD), was 530 mg/cm3, on MSC treated animals, and 553 mg/cm3 on control group of 3 animals where non critical size defects were treated with iliac crest autologue bone graft. Activity of the Alkaline Phosphatase enzyme were measurement on 0.5,14,21,30 day and increased activity were detected day 14on animals treated with bone marrow MSCs compared with day 30 on iliac crest treated animals. That results indicates strong osteoinduction activity of the experimental bone marrow MSCs models exposed to the rhBMP-7 factor. Comparing ALP activity, that model showed superiorly results than control group. That result initiates us in opinion that MSCs alone should be alternative for the autolologue bone transplantation and* in vitro *study we isolated singles MSCs from the bone marrow of rat's tibia and femora and cultivated according to the method of Maniatopoulos et all. The small initial colonies of fibroblast like cells were photo-documented after 2 days of primary culture. Such isolated and cultivated MSCs in future studies will be exposed to the growth factors to differentiate in osteoblast and indicate their clinically potential as alternative for conventional medicine and autologue bone transplantation. That new horizons have potential to minimize surgery and patient donor morbidity, with more success treatment in bone regenerative and metabolism diseases.*

Key words: *mesenchymal stem cells, regenerative medicine, bone regeneration*

Introduction

Bone marrow contains at least two, and likely more[1,2], discernable stem cell populations. Besides the hematopoietic stem cell which produces blood cell progeny, a cell type termed Mesenchymal Stem Cells (MSCs) or stromal also exists in marrow. MSCs were first recognized in bone marrow as a non-hematopoietic stem cell by a German pathologist, Julius Cohnheim in 1867[3]. The isolation and exploration on the differentiation potential of MSCs have been an issue of research for more than three decades[3].

Human MSC have been shown to differentiate *in vitro* into various cell lineages including neuronal cells[4,5], as well as cartilage, bone, and fat lineages[6]. Also MSCs have been induced to form hepatocytes in culture[7] and liver-specific gene expression has been induced *in vitro* in human bone marrow stem cells[8].

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Numerous references now document the ability of these adult stem cells to contribute to regeneration of cardiac tissue and improve performance of damaged hearts. In animal studies, for example rat[9], mouse[10] and human[11] stem cells have been identified as integrating into cardiac tissue, forming cardiomyocytes and/or cardiac blood vessels, regenerating infarcted heart tissue, and improving cardiac function. In mice, bone marrow derived stem cells injected into old animals seem capable of restoring cardiac function[12], apparently through increased activity for cardiac blood vessel formation. The evidence has led numerous groups to use bone marrow derived stem cells in treatment of patients with damaged cardiac tissue[13,14]. Results from these clinical trials indicate that bone marrow derived stem cells, including cells from the patients themselves, can regenerate damaged cardiac tissue and improve cardiac performance in humans. Tateishi-Yuyama et al.[15], have shown that bone marrow derived stem cells from the patients themselves can improve blood circulation in gangrenous limbs, in many cases obviating the need for amputation.

Rodents (rabbit, rat and mouse) MSCs are classically obtained from the femurs and tibias, by flushing the marrow out of the bones with culture medium and transferring the resultant cell suspension in culture[16]. Human MSCs can be similarly obtained from healthy volunteers by taking aspirates of bone marrow from the iliac crest and expanding on tissue-culture plastic[17]. The traditional isolation method relies on the fact that MSCs selectively adhere to plastic surfaces, whereas hematopoietic cells do not and can therefore be removed through medium changes, by serial passaging and after a number of passages the culture is enriched in the self-renewing fraction, the stem cells[18].

In this study we want to show *in vivo* capacity of the bone marrow MSC to produce bone in non critical size defects of rabbit's mandibles. In second part of study we would like to address *in vitro* isolation and cultivation of the MSC derived from the bone marrow, as potential cell transplantation model for bone regeneration and alternative for conventional bone grating.

Material and Methods

For *in vivo* study we used total of 6 White New Zealand Rabbits. Animals were anesthetized with injection of the 1mg. Ketamine hydrochloride (Veterinary Ketalar) administered intramuscularly. With sub mandible approach, using dental micro motor and drill, unilaterally non critical defect of mandibles were created on all animals. Untreated, such kind of defect would healing incomplete with fibrous and scar tissue, pseudoarthrosis and dysfunction. Defect of mandible was firmly fixed bicortically with titanium mini plate. On three animals defects were treated with autologue bone marrow obtained from the resection particle of mandible and mixed with the growth factor recombinant human Bone Morphogenetic Protein-7 in concentration of 100 micrograms in collagen as carrier. On the control group of other three animals defects were treated with bicortical autologue bone graft from crest iliac.

Results were detected by measurement Alkaline Phosphatase (ALP) enzyme activity on 5, 14, 21, 30 postoperative days, and by 3D C-T images with slices 1mm thick, taken after 30 days. That 3D CT images were analyzed for the Bone Mineral Density (BMD, mg/cm3) using GEANIE 2.0 software (BonAlyse Ltd.). During C-T recording animals were anesthetized with 1 mg Ketamine.

In vitro study the stem cells were isolated from the bone marrow of rat's tibia and femora and prepared according to the method of Maniatopoulos et al.[19]. The animals 5–6 week old were used, because the donor age affects the amount of mineralized tissue (20). Both sites femora and tibia were aseptically removed after sacrifice under chloroform anesthesia asphyxiation according to Institutional Animal Care and Use Committee (IACUC) guidelines. The adherent soft tissues were thoroughly debrided and the bone is washed 5 times with phosphate buffered saline (PBS). After the epiphyses were removed, an 18-gauge needle, attached to a 10-ml syringe was used to bore a small opening through the growth plate on the distal end of the femora and the proximal end of the tibiae. A 10-ml syringe fitted with a sterile 18-gauge needle was filled with alpha minimal essential medium supplemented with 10% fetal bovine serum (FBS). The needle was inserted into the opening previously prepared, and 5ml of medium was ejected to expel the bone marrow from the medullary canal. Marrow samples were collected suspended and inoculated in the 75 cm^3 cell flask with 15 ml medium. The cells are stored and maintained at 37C in a humidified atmosphere, consisting of 95% air and 5% CO2. To prevent bacterial contamination we use Penicillin 50UI/l, Streptomycin 50 micrograms/ml, Fungizone 0.3 micrograms/ml and Gentamicine Sulphate 50 micrograms/ml, dissolved in the medium before filtration. The initial colonies were visualized and photo-documented.

Results

Alkaline phosphates activity

Mean values of ALP activity observed preoperative and 5^{th}, 14^{th}, 21^{th}, 30^{th} postoperative day are present in Table 1. Significant higher mean values of ALP activity

TABLE 1
MEAN VALUES OF ALP ACTIVITY MEASUREMENT ON DEFECTS TREATED WITH BMMSC&rhBMP-7 (3 ANIMALS) AND BICORTICAL BONE GRAFT TREATED SITES (3ANIMALS)

bicortical bone graft	29.02	27.49	22.65	27.03	54.38
BM MSC&rhBMP-7	41.3	18.48	38.49	44.03	48.72
Days	0	5	14	21	30

BMMSC&rhBMP7- bone marrow mesenchymal stem cells and recombinant human bone morphogenetic protein 7

TABLE 2
TIME COURSE INTERVAL OF THE DIFFERENCES MEAN VALUES OF ALP ACTIVITY BMMSC&rhBMP-7 AND BONE GRAFT TREATED SITES

Significances	t df Sig	t df Sig	t df Sig	t df Sig	t df Sig
Bicortical bone graft	2.4/2/0.131	6.8/2/0.020	7.8/2/0.016	19.1/2/0.003	10.0/2/0.010
BM MSC&rhBMP	12.9/2/0.006	16.9/2/0.003	4.0/2/0.050	5.1/2/0.035	12.8/2/0.006
Days	0	5	14	21	30

BMMSC&rhBMP7- bone marrow mesenchymal stem cells and recombinant human bone morphogenetic protein 7

were detected 14th postoperative day on animals treated with BMMSC& rhBMP-7/ACS with 0.050 level of significance, than in control group of bone graft treated sites 30 day with 0.010 level of significance (Table 2).

C-T and BMD evaluation

Examinations C-T scans and analyzing BMD using GENAUINE 2.0 program are present in Figure 1a and b.

Average values of the BMD determinate by the GEANIE 2.0 BonAlyse program 3 animals treated with the BM MSC&rhBMP-7 and 3 animals treated with bicortical bone graft, 30th postoperative day are showed in Table 3.

In vitro *isolation MSCs from bone marrow*

The small initial colonies of fibroblast like cells were photo-documented after 2 days of primary culture what is showed in Figure 2. There are present initial colonies of the stem cells attached for the floor of flask. They are of airy consistence, star like shape with nucleus in the middle. Around them are roundish truss like confluent

a)

b)

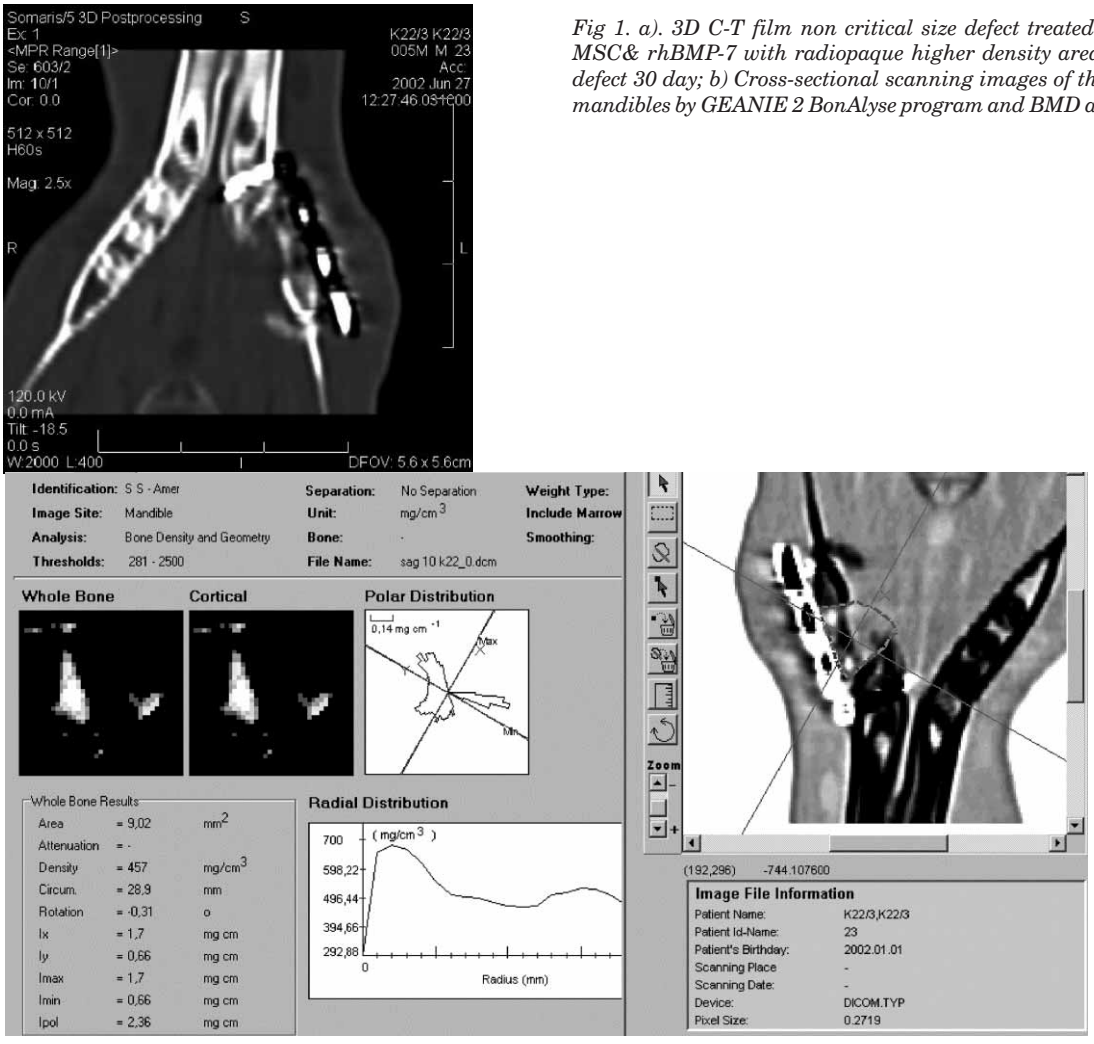

Fig 1. a). 3D C-T film non critical size defect treated with BM MSC& rhBMP-7 with radiopaque higher density area entire of defect 30 day; b) Cross-sectional scanning images of the resected mandibles by GEANIE 2 BonAlyse program and BMD analyzing.

TABLE 3
MEAN VALUES BMD DAY 30 OF BMMSC&rhBMP-7 AND BONE GRAFT TREATED SITES

	BMD values	Std. deviation	St. Error Mean
bicortical bone graft	530.66	53.26	30.75
BMSC&rhBMP-7	553.66	115.37	66.60

BMMSC&rhBMP7- bone marrow mesenchymal stem cells and recombinant human bone morphogenetic protein 7, BMD- bone mineral density

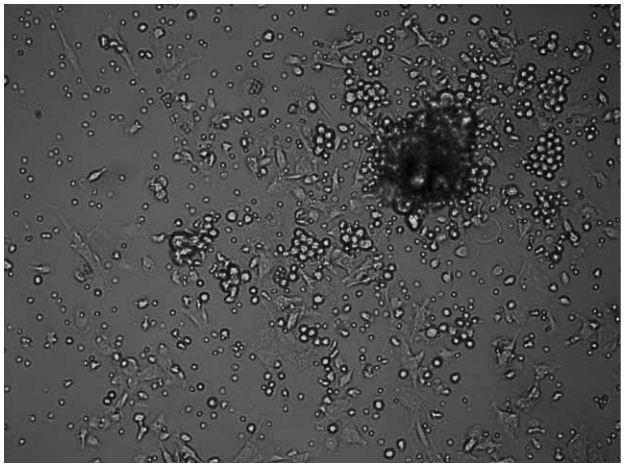

Fig 2. Two days fibroblast like cells colony together with some hematopoetic cells

hematopoietic cells, which during the experiment should be eliminated during media changes by aspiration and flushing, and stem cells will continue to reproduce and cultivate.

Discussion

This *in vivo* study showed that bone marrow exposed to the growth factor BMP-7 has strong osteoinductive response detected by ALP activity and BMD measurements. Even that, showed more superior osteoinductive effect than gold standard in bone reconstruction, bicortical bone graft, detected by significantly increase ALP activity 14 day, compared with 30 day on bone graft sites. The reason for that should be fact that bone marrow is contains of the stem cells[20,21]. Also we suppose that excessive bone formation observed 30th day on CT film originated due excess response stem cells to the rhBMP-7, and their osteoinduction signaling mechanism.

Judging from the results of this in vivo experiment, our next intention was to isolate stem cells from bone marrow, as future cells transplantation model. The important technical point was that the culture medium containing fetal bovine serum.

Most cells which do not attach to the culture dish surface were removed by aspiration at the first medium change, 24 hours after cell inoculation, and a very small number of fibroplastic stem cells remain. When the cells are prepared in this way and cultured under the above condition, the majority of the hematopoetic cells die and stromal fibroplastic colonies are formed. Marrow cells give rise to fibroplastic MSC colonies in culture and each colony is derived from a single cell, what was in accordance with method described by Friedenstein[3].

Fibroplast MSC colony forming cells are termed a fibroplastic colony forming unit (CFU-F). Ergise et al.[22], reported that the bone marrow of a 4-mount old rat form more fibroblastic colonies than the bone marrow of a 21-month old rat, indicating a decrease in CFU-Fs in bone marrow with aging. Some CFU-Fs, which consist of osteoprogenitor cells, have the capacity to produce bone like mineralized nodules. Thus, we can estimate the number of osteoprogenitor cells in bone marrow tissue by counting the mineralized – nodules in this culture system. Also we used in study mice between 4 and 6 week old to obtain the optimum sample for obtaining CFU-F MSC.

BM MSC expansion potential, ease of isolation, compatibility with multiple delivery methods and most importantly their immunosuppressive properties have generated great expectations in cell-based therapeutics[23,24]. The bone graft transplantation should be replace in future and bone harvesting with their morbidity and necessarily for adding operative procedures become history.

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IN VIVO OSTEOINDUKTIVNI EFEKT I *IN VITRO* IZOLACIJA I KULTIVACIJA MEZENHIMALNIH MATIČNIH STANICA KOŠTANE SRŽI

S A Ž E T A K

Koštana srž sadrži stanice koje se zovu mezenhimalne matične stanice (MMS) koje su prvi put identificirane u koštanoj srži od strane njemačkog patologa Julija Cohnheima 1867 godine. Utvrđeno je da MMS imaju potencijal da se diferenciraju *in vitro* u različite tipove stanice kao što su osteoblasti, hondroblasti, mioblasti i adipoblasti. Ciljevi ovog istraživanja su da se *in vivo* pokaže sposobnost MMS iz koštane srži da proizvedu novu kost kod kirurški stvorenih defekata nekritične veličine kod novozelandskih kunića. U drugom dijelu studije da se izoliraju *in vitro* MMS iz koštane srži, kao potencijalni model stanica u regeneraciji kosti. *In vivo* studija je pokazala postojanje novog dijela kosti na 3D CT rekonstrukciji. Nakon 30 dana kod sve 3 životinje sa defektom nekritične veličine tretiranih sa MMS iz koštane srži izložene su ljudskom koštanom morfogenskom proteinu 7 (rhBMP-7). Prosječna vrijednost mineralne gustoće kostiju (BMD) iznosila je 530 mg/cm3 kod s MMS tretiranim životinjama, a 553 mg/cm3 u kontrolnoj skupini u kojoj su 3 životinje tretirane zbog defekata nekritične veličine sa autolognim graftom ilijačne kosti. Aktivnosti enzima alkalne fosfataze su mjerene tokom 0, 5, 14, 21, 30 dana te je povećana aktivnost otkrivena 14 dana kod životinja tretiranih sa MMS iz koštane srži u usporedbi s 30 danom kod životinja liječenih autolognim graftom. Ovaj rezultat ukazuje na snažan osteoindukcioni efekat djelovanja eksperimentalnog modela MMS koštane srži izloženog rhBMP-7 faktoru. Upoređujući ALP aktivnosti ovaj je model pokazao superiornije rezultate u odnosu na kontrolnu skupinu. Ovaj rezultat inicira na mišljenje da bi MMS metoda trebala biti alternativa za autolognu transplantaciju, te su u *in vitro* ispitivanju izolirane pojedinačne MMS iz koštane srži tibije i femura štakora i kultivirane prema metodi Maniatopoulos et al. Male početne kolonije stanica fibroblasta su fotografski dokumentirane nakon 2 dana od primarne kulture. Takve izolirane i kultivirane MMS će tokom studija u budućnosti biti izložene faktorima rasta i diferencirati se u osteoblaste te ukazati na njihov klinički potencijal kao alternativu za metode konvencionalne medicine i autolognu transplantaciju kosti. Ovi novootvoreni horizonti ukazuju na potencijal minimiziranja kirurških zahvata kao i morbiditeta bolesnika i donatora, uz više uspjeha u liječenju kod regenerativnih i metaboličkih oboljenja kostiju.