Local and Systemic Administration of Mesenchymal Stem Cells in Regeneration of Mandibular Defects: An Experimental Study

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Introduction: Mesenchymal stem cells (MSCs) have been utilized as an aid in regeneration of bone defects however due to the poor homing ability via systemic injection, local administration, genetic manipulation has been used more. The present study aimed to compare the local and systemic application of MSCs in regeneration of bone defects. **Materials and Methods:** Sixty Wistar male rats were used in this study. Circular 5×5 mm bone defects were created in the angle of the mandible. Autogenous bone marrow mesenchymal stem cells (BMMSCs) were obtained and culture expanded. Green fluorescent positive (GFP+) transfection has been performed for the possible detection of the cells in the healed bone. The rats were randomly treated in four groups; 1. Beta tricalcium phosphate (β-TCP) scaffold seeded with MSC (β-TCP+MSC), 2. Intravenous MSC injection with β-TCP scaffold in the defect (MSC), 3. β-TCP without cell therapy (β-TCP) and 4. Control defects that left empty. The rats were euthanized after 8 weeks. New bone formation (BF) were investigated by hematoxylin and eosin staining. **Results**: Localized cell therapy (β-TCP+MSC group) showed the highest healing rate (BF= 54.05±2.39%) followed by MSC systemic injection (BF= 22.69±3.87%) *(P≤0.05)*. Inflammatory cells infiltration was evident dominantly in systemic injection cell group. Immunohistchemical analysis showed existence of the MSCs around the defects. GFP+ cells were mainly detected in the defect in β-TCP+MSC group while few GFP+ cells were detected in the MSC systemic group. **Conclusion:** local application of the MSCs with synthetic scaffold showed better results than intravenous administration of MSCs in treatment of rat mandible bone defects.

Keywords: Mesenchymal Stem Cells; Bone Regeneration; Regenerative Medicine; Tissue Engineering

Introduction

Reconstruction of craniofacial defects in congenital deformities, post- traumatic and post-operative bone surgery has been the topic of bone regeneration (1-3). The size of bone defects is very critical in bone regeneration; hence the replacement or regeneration of lost bone is not always successful (4). In according to rising demand for orthopedic and maxillofacial surgical procedures as a consequence of population aging and increase in chronic diseases, new strategies for bone regeneration are needed. To date, using the primary bone has been the main approach for replacing missing bone. Autogenous bone graft is the golden standard in treatment of these defects (5,6) but the limited sources and associate morbidities (7) have overshadowed its application. Although the alternative choices including allografts and synthetic materials were utilized to overcome these limitations, these methods have faced some drawbacks like immunological response and low regenerative properties in large defects (8,9).

Adult or postnatal stem cells represent promising candidates for regenerative therapy, since they have the potential to replicate in an undifferentiated state as well as to differentiate along committed lineages. Mesenchymal Stem cells (MSCs) are

found throughout the body and numerous extraction protocols have been established for different tissues (e.g., umbilical cord, adipose tissue, skeletal muscle, deciduous teeth, and other tissue) (10-12). For more than 40 years, bone marrow-derived stem cells have been the most frequent sources for cell therapy. These cells can be isolated from bone marrow and from bone chips (cortical or trabecular bone). If these cells seeded onto or cultivated on calcium phosphate ceramic matrices *in vitro*, they can induce bone formation *in vivo* (13-15). MSCs are multipoint cells that can be differentiated to osteoblasts and chondroblasts 16. MSCs has been used with different kind of scaffolds to enhance healing in bone defect (16,17). The large bony defect had less chance of complete healing 18. Due to the associated morbidities with local application (19) and concerns regarding cell viability following seeding on scaffolds (20), their systemic infusion has also been the topic of interest. A key steps in bone healing is homing of MSCs (21). Kumagai *et al,*. showed very slight contribution of circulating cells in direct repair of the injured bone (22). One clinical trial used intravenous MSCs to treat osteogenesis imperfect in children (23). Several studies have demonstrated that systemic application of MSCs leads to homing and enhanced repair in the defect (24,25), while others have demonstrated that majority of injected cells home in lungs (26) and fail to participate in regeneration. The present study aimed to assess the potential of local and systematic application of MSCs in healing of mandibular bony defects.

Materials and Methods

Animal models

Sixty 7-week old Wistar rats weighing 400-450 gr were used following approval by the Institutional Animal Care and Use Committee (IACUC) of the Tehran University and accordant with the standards of Association for Assessment and Accreditation of Laboratory Animal Care. They were individually housed in plastic cages throughout the study in a monitored situation (21°C, 12:12 light cycle). The animals were fed with standard laboratory rat food pellet diet and water was available ad libitum.

Cell isolation and implant preparation

In an aseptic conditions and with general anesthesia, 100 µL bone marrow was aspirated from the shaft of tibia bone with a 20-gauge biopsy needle in a heparinized tube and the samples were diluted 1:3 in Dulbecco modified Eagle medium (DMEM; Gibco, Paisley, UK). On day one, non-adherent cells were discarded and adherent cells were washed with phosphate buffered saline (PBS, Gibco, Paisley, UK). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, Paisley, UK), 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco, Paisley, UK). The cells were cultured until the third passage with 1×10^6cells/mL concentration. One day prior to transplantation, 4×106 third subculture MSCs in 0.1 mL DMEM were placed on top of 150-500 µL β-TCP granules (Cerasorb, Germany) for at 37°C.

Cell characterization

Flow cytometry (ATTUNE® Flow cytometer (Applied Biosystems) was performed according to the protocols as follow: Fluorescen isothiocyanate (FITC)-conjugated monoclonal antibodies against CD34 (Mittenyi Biotech, Bergisch-Gladbach, Germany), CD13, CD105, CD29, and CD44 (BD Bioscience, San Diego, CA) were used at 2 mg/mL concentration at 4°C for 30 minutes. Cells painted with FITC-labeled mouse immunoglobulin G were considered as the negative control. Cells were washed twice with PBS and 1% paraformaldehyde was used for fixation. Presence of 99% fluorescent-labeled cells were considered as positive specimen.

GFP transduction

Virus packaging was performed by a lentiviral GFP expressing vector and helper vectors (psPAX2 and pMD2.G). Then, supernatant media containing lentiviral viruses were harvested 48 h post-transfection. Transductions of rat-BMMSCs were carried out at MOI of 20 in the presence of 8 µg/ml polybrene. After 4 days, the efficacy of transduction was evaluated by flow cytometric analysis using propidium iodide (PI) to identify non-viable cells. A flow cytometer (Applied Biosystems) was used to detect viable populations of transduced [GFP (+)] and non-transduced [GFP (-)] cells. Gating parameters were set with 3 control MSC groups: non-infected; PI stained, apoptotic; and GFP-infected cell.

In vitro osteogenic differentiation

Passage-3 MSCs were cultured in osteogenic medium to assess the osteogenic potential of isolated MSCs. The osteogenic

medium consisted of DMEM, 50 µg/mL ascorbic 2-phosphate (Sigma Aldrich, St. Louis, MO, USA), 10 nmol/L dexamethasone (Sigma Aldrich, St. Louis, MO, USA), and 10 mmol/L β-glycerol phosphate (Sigma Aldrich, St. Louis, MO, USA). Following three weeks, the cells were fixed with 4% formalin for one hour and rinsed with PBS (Gibco, Paisley, UK). Mineralization was assessed following 5 minutes staining with 40 mmol/L Alizarin Red S, pH 4.2m.

Scanning electron microscopy

Scaffolds were fixed in 2.5% glutaraldehyde at 4°C for 24 hours and then washed with PBS. Dehydration was performed with graded series of ethanol (30%, 50%, 80%, and 100%) and vacuum drying. The samples were coated with gold and visualized at an accelerating voltage of 20 kV by scanning electron microscopy (Akishima Tokyo, Japan).

Surgical procedure

General anesthesia was induced in the rats by intramuscular injection of 2 mg/kg xylazine-HCl (xylazine 2%, Alfasan International BV, Woerden, the Netherlands) followed by local anesthesia by Lidocaine 2% (Daroupakhsh, Tehran, Iran) in the surgical site. Mucoperiosteal flaps were placed in the right buccal vestibule and 5×5 mm bone defects were created on the buccal surface of the mandible by the use of a round surgical bur (Meisseinger, Dusseldorf, Germany) in all samples. The rats were randomly allocated to four groups: 1. β-TCP scaffold loaded with MSCs (β-TCP+MSC), 2. MSC systemic injection (MSC), 3. β-TCP in the defect without cells (β-TCP) and 4. Defect without any cells or scaffolds (control). The flaps were closed in layers by resorbable sutures (Vicryl 3.0; Ethicon GmbH & Co. KG, Norderstedt, Germany). Twenty-four hours following the surgery, 1×10^6 cells were injected through the tail vein in MSC group. Ten mg/kg diazepam and 10 mg/kg enrofloxacin intramuscular injections were used for post-surgical pain and infection control.

Histological and histomorphometerical evaluation

The samples were euthanized after eight weeks with anesthetic over dose and the mandibles were removed. The specimens were fixed for 5 to 7 days in 10% buffered formalin and were decalcified in formic acid and sodium citrate for 24 hours. Subsequently the samples were washed with tap water, dehydration was performed by ascending concentrations of ethyl alcohol and cleared in xylene (Sigma Aldrich, St. Louis, MO, USA) and infiltration was performed by paraffin. 7 µm serial sections were cut by a microtome from the defect center parallel to the midsagittal suture and were stained with hematoxylin and eosin (H&E). Bone formation was assessed by light microscope (E400, Nikon, Tokyo, Japan) with ×400 magnification and analysis was performed by histomorphometric software (IHMMA-Veril, SBMU, Iran).

Immunohistochemistry

Tissue sections were analyzed immunohistochemically to detect the GFP antigen. 5µm cuts of the tissue sections were incubated with the primary antibody Anti-GFP (Novacastra Laboratories Ltd., Newcastle, England) overnight at 4 °C. Then secondary antibodies were added. Afterwards Strept AB/HRP (DAKO) was added for binding actual dye AEC+ (DAKO) in accordance with the instructions of the manufacturer. In the negative control sections, the primary antibody was omitted. This way, the original fluorescence was green, but the immunostained GFP was brown.

Statistical Analysis

Mean percentage of new bone formation in each animal were compare by one-way analysis of variance (ANOVA) in SPSS 22.0 (SPSS Inc., Chicago, IL). Significance level was considered 0.05 or less.

Results

Osteogenic capability of isolated rat-BMMSCs

In vitro evaluation of osteogenic differentiation of rat MSCs were studied by alizarin red staining and the results confirmed osteogenic differentiation of MSCs (Figure 1).

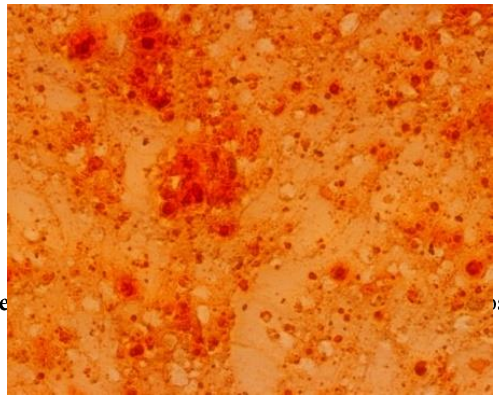

Figure osteoblasts

Flow cytometry analysis

The flow cytometry analysis results demonstrated that the cells were positive for CD29, CD44, CD105 and negative for CD34 (data not shown).

SEM analysis

SEM micrographs demonstrated MSC scattering through the scaffold pores and adherence was demonstrated by cellular pods and attachments (Figure 2).

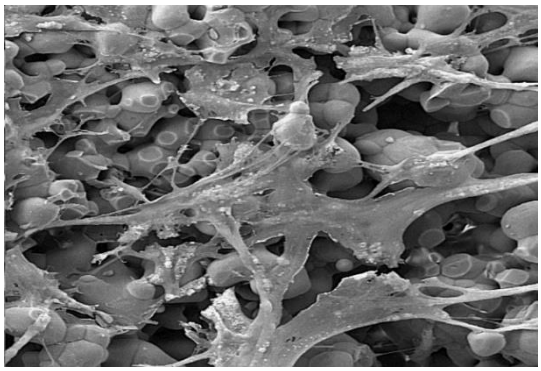

Figure 2: SEM image of the rat-BMMSCs seeded on β-TCP scaffold

Histological and histomorphometrical analysis

In the present study 60 rat mandibular defects were histologically and histomorphologically evaluated. The defect sites exhibited variable healing between groups, ranging from scattered bone formation in groups β-TCP+MSC, MSC, and β-TCP to almost lack of bone formation in the control group (Figure 3 A-D).

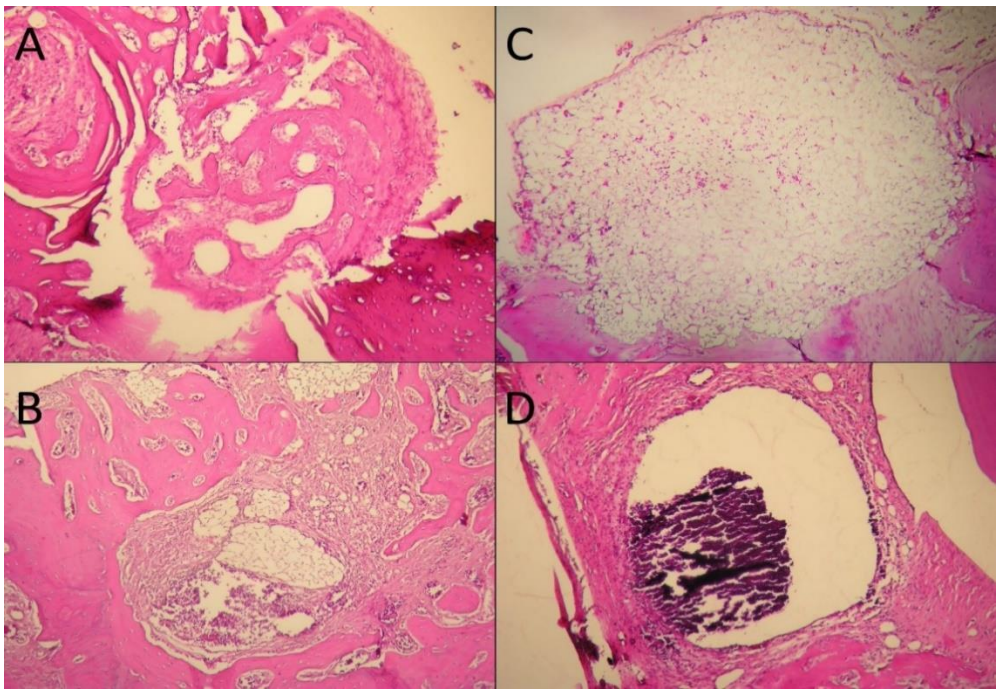

Figure 3: Histologic section of defect representing normal bone with few inflammatory cells; A) β-TCP scaffold seeded with MSC (β-TCP+MSC); B) Intravenous MSC injection with β-TCP scaffold in the defect (MSC); C) β-TCP without cell therapy (β-TCP); D) Control defects that left empty

new bone formation with dense arrangement of the osteocytes was detected in the periphery of scaffolds in β-TCP+MSC, MSC and β-TCP groups which suggested bone formation from the periphery to the center of the scaffold and it consisted of lamellar and trabecular bone. In the β-TCP+MSC group, the scaffold pores were occupied by cuboidal active osteoblasts with woven bone formation as well. There was no sign of inflammation in groups β-TCP+MSC, β-TCP and control, however inflammatory cells were detected within the defects in the MSC group more fibro-fatty degeneration with the remnants of the scaffold. The mean of regenerated bones was significantly different among the four study group *($p<0.05$)*.

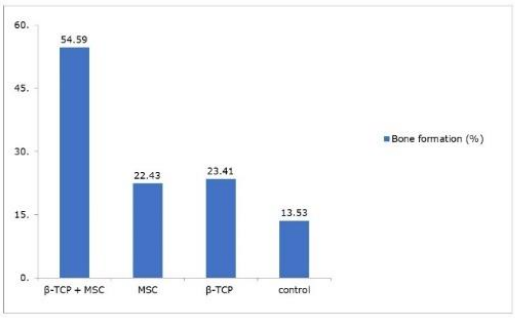

Figure 4: Mean of bone formation among the four study groups

Histomorphometry analysis results are demonstrated in (Figure 4).

Immunohistochemistry analysis

The IHC evaluations was performed and comparing to the MSC group, greater GFP+ cells were detected in β-TCP+MSC group mainly in the periphery of the defect. There was no GFP+ cells detected in β-TCP and control groups (Figure 5).

Discussion

Local application of stem cells has been extensively utilized in treatment of bony defects (16,20,27,28) but due to minimally invasive and ease of procedure, systemic injection of stem cells has been in the spotlight. In the present study, BMMSCs were delivered to the host by seeding on the scaffold or injecting through the veins. The maximum bone regeneration was observed in the local administration and the control group demonstrated the least bone formation. It is demonstrated that infusion of ex vivo expanded BMMSCs leads to lodgment of these cells mainly in lungs, liver, heart and spleen 26. Accordingly, it is assumed that GFP+ cells could be detected once sections from lungs were obtained, however the literature is controversial in this regard and some previous studies had suggested enhanced tissue repair via BMMSC homing 24 while others illustrated few GFP+ cells at the defect despite the significant fracture healing in systemic injection (29).

Intra-bony injection of BMMSCs to the femur of osteoporotic rats has demonstrated presence of GFP+ cells at the site and increased bone regeneration, though the cells were injected intra-bony into the femur and histologic evaluations were confined to the same bones and systemic interaction of the cells was not studied (30). On the contrary, in another study systemic and local injection of MSCs in osteoporotic rats did not demonstrate any positive effects (31) which may be due to the timing of disease modeling and stem cell infusion. Furthermore, it is demonstrated that systemic injection of adipose derived stem cells in non-critical sized defects accelerates healing comparing to local injection (25). Moreover, Miya Kanazawa *et al.*, reported that local MSC injection was more ineffective than systemic MSC injection at enhancing peri-implant epithelium (PIE) sealing around titanium implants in a rat oral implantation model (32).

Recently it has been hypothesized that T-cells inhibit MSC mediated bone formation by down-regulating RUNX-2 and enhancing TNF-α and IFN- γ levels. On this account, treating nude mice with pan-T cells and systemic infusion of Foxp3+ regulatory T cells (which induce immune tolerance) markedly increased MSC mediated bone regeneration (33) and this may serve as a decent reason for incomplete healing even in our

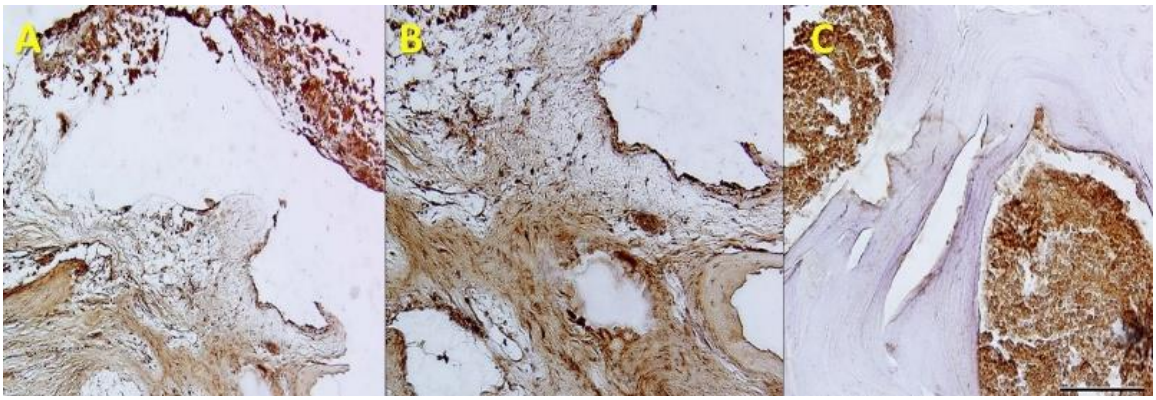

Figure 5: IHC illustration of bone defect in systemic MSC injection. The brown cells (arrows) illustrate GFP+ cells which have migrated to the periphery defect and n4o GFP+ cell is detected in the sound surrounding bone; A and B) Distribution of GFP+ cells (brown cells) through the bony defect in the local application of the cells (β-TCP+MSC); C) lack of GFP+ cells in the control group

locally administered β-TCP+MSC group. This hypothesis was further accredited when greater bone was generated by alginate coated MSCs (34) and significant healing was observed in periodontal defects subsequent to local injections with decrease in TNF-α, IFN- γ, and IL1β 35. Recently, BMMSCs pretreated with acetylsalicylic acid (ASA) were utilized in a study to increase bone healing in rat model for periodontal defects. The results showed that level of inflammatory cytokines like TNF-α and IL-17 decreased while the level of suppressing cytokine like IL-10 increased. So, they suggested that co-application of ASA and BMMSCs may be a novel strategy for periodontal bone regeneration (36).

Furthermore, infusion of BMMSCs prior to transplantation of cell loaded scaffolds has resulted in greater bone healing. It is assumed that systemic infusion of BMMSCs enhances bone regeneration via their immunomodulatory properties by decreasing TNF-α, IFN- γ and promoting CD4+ CD25+ Foxp3+ cells; however the injected cells may fail to home in the defects to generate bone directly (37). On this account, our findings are concurrent with previous studies which suggest scarce cell homing and possible inhibitory effect of immune system in stem cell mediated bone regeneration, however we could have validated the underlying mechanism by measuring TNF-α and IFN- γ levels and this may serve as the shortcoming of the present study.

Conclusion

In conclusion, systemic application of BMMSCs does not seem to enhance bone regeneration in critical sized bone defects while its immunomodulatory property in co-application with cell seeded scaffolds needs to be meticulously addressed in future studies.

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Conflict of Interest: 'None declared'.

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