Mesenchymal Stem Cell Therapy for Treatment of Craniofacial Bone Defects: 10 Years of Experience

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Submitted: 2015-08-24; Accepted: 2015-10-22; DOI: 10.7508/rrr.2016.01.001

Introduction: Cell delivery in treatment of bone defects has been introduced to promote tissue healing in the recent years. However, no general consensus has been reached regarding the outcome of regenerative medicine for this purpose. The aim of this study was to review our 10 years of experience in application of mesenchymal stem cells (MSCs) in craniofacial bone defects.

Applied Methodology: Iliac bone marrow, dental pulp and buccal fat pad were selected to harvest MSCs. Flow cytometric analysis, RT-PCR and differentiation staining including Alizarin red, Oil Red O and Toluidine blue were used to identify MSCs. Four groups of bone substitutes were used for cell delivery: synthetic scaffold [beta-tricalcium phosphate (B-TCP) and hydroxyapatite/tricalcium phosphate (HA/TCP)], xenograft [natural bovine bone mineral (NBBM)], allograft [freeze-dried bone (FDBA), demineralized freeze dried bone] and composite [polycaprolactone/TCP (PCL-TCP), demineralized freeze-dried bone/calcium sulfate]. Rat and rabbit calvaria, dog mandible, rabbit tibia sinus and alveolar cleft defects in human were used as the study models. Histomorphometric and radiomorphological analysis were used to determine new bone formation. Outcomes: Cell-treated groups showed greater new bone formation than cell-free group in all studies. Synthetic scaffolds showed better cell attachment according to scanning electron microscopy (SEM) results. In rat calvarial model, B-TCP loaded with MSCs showed better results than scaffolds carrying platelet rich plasma (PRP). NBBM showed less promising results both in dog mandible and ectopic bone formation in the masseter muscle. FDBA block fixed over a supracrestal defect in dog mandible showed 50% less new bone formation when compared with PCL-TCP as a carrier. Conclusion: More convergence studies with similar protocols of cell cultivation, culture, seeding and delivery should be done in the field of regenerative medicine for better generalizability of results for clinical setting.

Keywords: Mesenchymal stem cells; Bone regeneration; Tissue engineering; Craniofacial bone

Introduction

Bone as a highly differentiated tissue in the body may suffer defects due to congenital diseases, trauma, tumors and senile atrophy. Despite many advances in bone regeneration, reconstruction of large bony defects continues to be beyond the reach. Despite the availability of a plethora of treatment modalities, bone substitutes and various clinical adjuncts, most clinicians and researchers continue to reach the same conclusion. Autogenous bone grafts are still the “gold standard” to which all other reconstructive procedures are compared. However, autogenous bone grafts have several significant limitations, namely limited availability of competent sites and inherent donor site morbidity (1-4). Over the past few decades, considerable advancements have been made in tissue and bone engineering procedures. Bone engineering procedures apply osteogenic cells, growth factors and scaffolds to promote bone formation. Various osteogenic cells, including mesenchymal stem cells (MSCs) can be used in bone engineering protocols. MSCs have been proven to have proliferation and osteogenic differentiation capacities (5-8). Similarly, previous studies have demonstrated that MSC-based bone augmentation procedures induce higher levels of bone formation compared to acellular treatments (9-12). In addition, co-transplantation of growth factors in these procedures might further promote bone regeneration (13-15). The purpose of this study is to review our 10 years of experience in application of MSCs in craniofacial bone defects.
Applied Methodology

Harvesting the MSCs

**Iliac bone marrow aspirate**

Two weeks before regenerative surgery, bone marrow aspirate (10-15 mL) was obtained from the posterior iliac crest. The aspirate was diluted at 1:3 in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Gibco, Paisley, UK). On day one, non-adherent cells were discarded and adherent cells were washed with phosphate-buffered saline (PBS) (Gibco, Paisley, UK) and then cultured in DMEM/F12 with antibiotics and 20% autologous serum.

**Dental pulp**

Vital third molars of a healthy adult subject indicated for extraction were extracted. Before extraction, the patient was asked to rinse 0.2% chlorhexidine mouthwash (Behsa® Co., Tehran, Iran) for one minute. After extraction, the teeth were immersed in saline solution containing antibiotics. The crown of each tooth was cut into several segments and the pulp was exposed. Dental pulp tissue was removed with a barbed broach. Dissected tissue was minced and incubated in 3 mg/mL type I collagenase and 4 mg/mL dispase in PBS (Gibco, Paisley, UK) at 37°C for 30 minutes. Then, the tissue fragments were immersed in α-MEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (Gibco, Paisley, UK) and placed in 25 cm² flasks. Flasks were incubated in humidified atmosphere with 5% CO₂ at 37°C. Culture medium was changed twice a week and after reaching the sub-confluent stage, the cells were removed by enzymatic digestion (0.25% trypsin–EDTA) and passaged. Third to fourth passage dental pulp stem cells (DPSCs) were subjected to the following experiments.

**Buccal fat pad**

Buccal fat pad was harvested from healthy donors via a vestibular incision distal to the maxillary second molar. The fat pad was exposed using blunt dissection while preserving the thin covering membrane; 3-5 mL of the fat was excised and transferred to a laboratory in DMEM. The tissue processing and cultivation of stem cells were done similar to the methods done for dental pulp. Adherent cells were expanded as monolayer cultures in 5% CO₂ at 37°C. Culture medium was changed twice a week and after reaching the sub-confluent stage, the cells were removed by enzymatic digestion (0.25% trypsin–EDTA) and passaged. Third to fourth passage dental pulp stem cells (DPSCs) were subjected to the following experiments.

Preparation of human serum

In human trials, human serum was used for cultivation of the cells. From each donor bone marrow, 20 mL of whole blood was collected in blood bags and quickly transferred into 10-mL vacutainer tubes without anticoagulants (BD, Plymouth, UK), and allowed to clot for 4 hours at 4°C to 8°C. Subsequently, the blood was centrifuged at 1800g at 4°C for 15 minutes. Serum was collected and filtered through a 0.2 mm membrane.

Identification of MSCs

**Flow cytometry analysis**

Fluorescence-activated cell sorting (FACS) was performed using standard protocols and quantification criteria. The criteria to distinguish positive from negative cells were set individually for each marker. Fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies against CD34 (Miltenyi Biotech, Bergisch Gladbach, Germany), CD44, CD29, CD105 and CD13 (BD Biosciences, San Diego, CA, USA) at a concentration of 2 mg/mL at 4°C were used for 30 minutes. The cells stained with FITC-labeled mouse IgG were used as negative controls. The cells were washed twice with PBS and fixed with 1% para-formaldehyde. Analysis was performed on 100,000 cells per sample and the positive expression was defined as the level of fluorescence greater than 99% of the corresponding value for unstained cell sample (11-14).

**Differentiation Staining**

**Alizarin Red staining (osteogenic differentiation)**

To induce osteogenic differentiation, cells from third passage were provided with DMEM medium supplemented with 50 mg/ml ascorbic 2-phosphate (Sigma Aldrich, St. Louis, MO, USA), 10 nM dexamethasone (Sigma Aldrich, St. Louis, MO, USA) and 10 mM glycerol phosphate (Sigma Aldrich, St. Louis, MO, USA). After 3 weeks, the cells were fixed with 4% formalin at room temperature, washed with 70% ethanol and stained with Oil Red solution in 99% isopropanol for 15 minutes.

**Oil Red staining (adipogenic differentiation)**

To induce adipose differentiation, third passage confluent cells were treated with a medium containing 100nM dexamethasone (Sigma Aldrich, St. Louis, MO, USA) and 50 mg/mL indomethacin (Sigma Aldrich, St. Louis, MO, USA). After three weeks, the cells were fixed for 1 h with 4% formalin and rinsed with PBS (Gibco, Paisley, UK). Mineralization of the extracellular matrix was visualized by staining with 40 mM Alizarin Red S at a pH of 4.2 for 5 minutes.

**Toluidine blue staining (chondrogenic differentiation)**

To induce cartilage differentiation, micro-mass culture system was used. For this purpose, 2.5×10⁵ third passage cells were
Table 1. Gene expression determined by RT-PCR for determination of differentiation potential of MSCs

<table>
<thead>
<tr>
<th>Cell lineage</th>
<th>Genes</th>
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<tbody>
<tr>
<td>Osteogenic differentiation</td>
<td>Osteopontin, Collagen I</td>
</tr>
<tr>
<td>Chondrogenic differentiation</td>
<td>Decorin, Collagen II</td>
</tr>
<tr>
<td>Adipogenic differentiation</td>
<td>LPL, PPARG2</td>
</tr>
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</table>

pelleted under 1200 g for 5 minutes and cultured in DMEM supplemented with 10ng/mL transforming growth factor-ß3 (TGFß3; Sigma Aldrich, St. Louis, MO, USA ), 10 ng/mL bone morphogenetic protein-6 (BMP-6; Sigma Aldrich, St. Louis, MO, USA), 50 mg/mL insulin transferrin selenium+ premix (Sigma Aldrich, St. Louis, MO, USA), 1.25 mg bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO, USA) and 1% FBS (Sigma Aldrich, St. Louis, MO, USA). After three weeks, the pellets were sectioned at 5 μm, stained with toluidine blue for 30 seconds at room temperature and observed under a light microscope.

**RT-PCR for gene expression**

The differentiation ability of the cells and some specific gene expressions were also studied by RT-PCR. For this purpose, total RNA was collected from the cells differentiated into 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, Waltham, Massachusetts, USA) 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, Waltham, Massachusetts, USA) according to the manufacturer instructions. Subsequently, RT-PCR was performed using 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, Waltham, Massachusetts, USA). 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.

**Cell Seeding**

A seeding concentration of 1.3×10^6 cells/mL was cultured on various bone grafts. Each layer of scaffold covering one of 24-well plates was seeded with 2×10^5 MSCs in 200 μL of culture medium. The plates were incubated for 1 hour at 37°C allowing cell adhesion; then, half of the samples received 1/500 of standard medium (containing α-MEM supplemented with 10% FBS, 1% antibiotic-antimycotic solution and 2 mmol/L L- glutamine). We used different incubation times for different types of bone substitutes including synthetic scaffold [beta-tricalcium phosphate (B-TCP) and hydroxyapatite/tricalcium phosphate (HA/TCP)], xenograft [natural bovine bone mineral (NBBM)], allograft [freeze-dried bone (FDBA), demineralized freeze dried bone] and composite [polycaprolactone/TCP (PCL-TCP), demineralized freeze-dried bone/calcium sulfate ]. The bone substitutes were mixed with cells and incubated for at least 24 hours. We also tested 48 hours to 14 days of incubation in some of our experiments. Cell attachment was assessed by SEM.

**Scanning Electron Microscopy (SEM)**

Scaffold surface topography, adherent cell morphology and extracellular matrix culture were assessed using SEM. The seeded scaffolds were rinsed with PBS twice, fixed in 2.5% glutaraldehyde for two hours, and then post-fixed in 1% osmium for another two hours. Then, samples were dehydrated in graded concentrations of alcohol (30, 50, 70, 90, 95, and 100% ethanol). Afterwards, the samples were air dried in a desiccator overnight, sputtered with gold and observed under a SEM (VEGA, TESCAN, Brno-Kohoutovice, Czech Republic) at 10 kV. Images of non-seeded scaffolds were also taken. Moreover, the size of micro- and macro-porosities of each scaffold was measured by Image J 1.46 computer software (Wayne Rasband, NIH, Bethesda, Maryland, U.S.).

**Application of MSCs**

Bone substitutes mixed with MSCs were transferred to bone defects in different animal and human models (9-19). Histological and histomorphometric analyses were done after 8-12 weeks according to the study protocols.
<table>
<thead>
<tr>
<th>Study</th>
<th>Treatments</th>
<th>Outcome</th>
<th>Comments</th>
</tr>
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<tr>
<td>A</td>
<td>BMSCs + PPF</td>
<td>Improved bone formation</td>
<td>Human, N/1, 2012</td>
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<tr>
<td>B</td>
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</table>
Outcomes

The SEM analysis showed lodgment of cells within the pores of the scaffolds (Figure 1). By increasing the time of incubation to seven days, the number of attached cells also increased (20). Synthetic materials showed more attachment that the bovine or human-derived substitutes (Figure 2) (20). The in vivo results of our studies were analyzed based on the type of defect and carrier for MSCs (Table 2).

Rat calvarial models

Through-and-through 5 mm circular defects were created in rat calvarium. Rat bone marrow mesenchymal stem cells (BMMSCs) delivered by β-TCP or NBBM (Bio-Oss; Geistlich, Wolhusen, Switzerland) were compared with groups receiving PRP-soaked scaffolds. New bone formation was measured histomorphometrically. The maximum bone formation was reported to be 2.53 mm in the β-TCP/MSC group six weeks after the surgery (11).

Rabbit models

Rabbit calvarial model

Two studies have been done on 8 mm rabbit calvarial defects. In the first experiment, MSCs were delivered by nano silica gel/hydroxyapatite (nanoHA) and new bone formation was measured histologically after six and 12 weeks post-surgery and in another one with the same protocol, MSCs were delivered by FDBA. In each study, there were four groups. Group 1: NanoHA granule; Group 2: Nano-HA+1 mL of autologous plasma rich in growth factors (PRGF); Group 3, Nano-HA+2 mL of culture medium containing 100,000 autogenous MSCs; Group 4, Nano-HA + 2 mL of culture medium containing 100,000 autogenous MSCs +1 mL autologous PRGF. Histomorphometric analysis at six and 12 weeks demonstrated significantly higher bone formation in group 4 (29.45% and 44.55%, respectively). Bone formation in groups 1, 2, and 3 was as follows: 11.35% and 32.53%, 29.10% and 39.74%, and 25.82% and 39.11%, respectively (14). In the second experiment, Nano-HA was replaced by FDBA. Histomorphometric analysis of the sections at six and 12 weeks post-operation demonstrated 14.6% and 20.31% osteogenesis in group 1, 14.35% and 28.44% in group 2, 17.75% and 31.33% in group 3 and 18.94% and 37.21% in group 4, respectively (16).

Rabbit tibial model

We used rabbit tibia as a study model for delivery of MSCs. Right and left tibiae of each rabbit were prepared, and a 3-mm protruding implant from the tibia was placed in each side. Particulate allogeneic bone/fibrin glue/MSCs combination was placed around test implants and particulate bone graft/fibrin glue around controls. Two months postoperatively, the animals were euthanized, and sections were prepared for histological analysis. The mean amount of vertical bone height was higher in the experimental group than the control group (2.09 mm versus 1.03 mm) (21).

Dog models

Ectopic bone formation

For evaluation of ectopic bone formation, a blunt pouch in the masseter muscle of a dog was created, bone marrow derived MSCs were implanted over the HA/TCP (group 1) and NBBM (group 2). The results showed 29.12 and 23.55% of new bone formation, respectively (19).

Mandibular defect in dogs

The effectiveness of MSCs in treatment of mandibular defects of
dogs was studied in three experiments. Six weeks after delivering dog BMMSCs with biphasic scaffold (HA/TCP) or NBBM (Bio-Oss) in a through-and-through 10-mm mandibular defect, new bone formation was reported to be 65.78% and 50.31%, respectively. The use of scaffolds without MSCs was accompanied by significantly lower bone regeneration (44.90% and 36.83%, respectively) (10). In another study we created a 10×20 mm supracrestal defect in the posterior mandible after extraction of the posterior teeth mimicking severe mandibular atrophy in edentulous patients. PCL-TCP block, which is a composite scaffold was used to carry the cells. The block was fixed with 1.2 mm micro screw (Jeil, Seoul, South Korea) to the underlying bone. Histomorphometric analysis eight weeks after scaffold implantation showed higher amount of lamellar bone in the test side (48.63%) than control side (17.27%) (17). In another study with the same protocol, freeze dried human derived bone block was used to deliver MSCs. Although in this study, MSCs were co-transplanted with recombinant platelet derived growth factor (rh-PDGF) the results showed only 21.52% of new bone formation (22).

**Human models**

We studied the application of MSCs in human subjects in three experiments. In the first study, we used HA-TCP loaded with BMMSCs in three cases of unilateral and four cases of bilateral sinus augmentation; dental implants were also placed in six patients, three months post-operatively. MSC-treated patients demonstrated adequate bone augmentation with 41.43% of new bone formation and an average bone height of 12 mm. These findings were based on histomorphometric and radiographic analyses three months after grafting. Radiographs 12 months postoperatively showed a 10.83-mm bone height increase. Twenty-eight of 30 implants (93%) were reported to be clinically successful at the six-month follow up (9).

In the second study, demineralized bone matrix was combined with calcium sulfate (DBM/CaSO4) and BMMSCs for secondary repair of alveolar cleft in two patients. Panoramic radiographs of two cases of grafted of alveolar cleft using MSCs displayed adequate bone fill and union of the segmental arches. Four-month quantitative measurements by tomographic scans showed a bone fill of 34.5% for the first case and 25.6% for the second case (12). In the third trial, we added PRGF to HA/TCP loaded with MSCs to treat alveolar cleft defects in three patients. Cone beam computed tomography showed 52% of new bone formation (13).

**Discussion**

The use of cells for treatment of organ disorders is a traditional goal in regenerative medicine. Culturing cells in the lab and then differentiating them to specific cell lines to either produce tissue in the lab or cause tissue healing in situ has always been an ultimate goal for researchers. The problems against tissue formation outside the human body include nutritional supply of the cells and collecting their waste materials. Bioreactors are gradually going to find their place in tissue engineering (22, 23). Diversity among the study protocols such as cell cultivation and differentiation, cell seeding on scaffolds, incubation period and delivery methods can affect the efficacy of regenerative medicine in bone reconstruction. In a recent article we could not detect any common method utilized by tissue engineering studies (7). We performed cell harvesting, culture, differentiation, cell seeding on scaffolds and delivery to sites with the same techniques in our experiments in the past 10 years. Synthetic scaffolds such as TCP showed better results in comparison to NBBM in 6 mm rat calvarial defects when delivering MSCs. The same result was obtained when PRP was added to the scaffold (11). Biphasic synthetic HA/TCP demonstrated better results in regenerating new bone in the dog through and through 10 mm mandibular defect than NBBM (10). HA/TCP also had better cell attachment when compared to NBBM under SEM (11). In two human studies performed for treating alveolar cleft defects, MSCs co-transplanted with HA/TCP caused more bone formation than DBM/CaSO4 co- transplanted with MSCs (12, 13). FDBA showed less bone formation than nano-HA with silica gel in 8 mm rabbit calvarial defects. FDBA and DFDBA showed the most promising results for cell attachments when investigated under SEM but in vivo studies showed different results. DFDBA loaded with MSCs was used around dental implants in rabbit tibia and revealed 2.1 mm vertical bone growth whereas no new bone formation was noted in the control cell-free group (21). FDBA as a block for treatment of posterior mandibular supracrestal defects in dogs was weaker in inducing new bone formation than PCL-TCP scaffold (26.63% versus 48.63%) (22). In vitro evidence showed proper cell attachment to various scaffolds but in vivo application demonstrated better results when MSCs were delivered by synthetic substitutes (Table 2).

**Conclusion**

To the best of our knowledge, bone regenerative potential of cells delivered to the defect site is not comparable to autogenous bone grafting although they can enhance bone healing when applied to bone defects. Future studies with the same protocols of cell cultivation, culture, seeding and delivery should be done in the field of regenerative medicine to reach from bench to bedside.

Conflict of Interest: ‘None declared’.
References


