Bone marrow mesenchymal stem cells enhance bone formation in orthodontically expanded maxillae in rats

Abdullah Ekizer; Mehmet Emir Yalvac; Tancan Uysal; Mehmet Fatih Sonmez; Fikrettin Sahin

ABSTRACT

Objective: To transplant bone marrow–derived mesenchymal stem cells (MSCs) into the intermaxillary suture after rapid maxillary expansion with the aim of increasing new bone formation in the suture.

Materials and Methods: Nineteen male Wistar rats were divided into two groups (control, n = 9; experimental, n = 10). Both groups were subjected to expansion for 5 days, and 50 cN of force was applied to the maxillary incisors with a helical spring. Pkh67\(^+\) (green fluorescent dye)–labeled MSCs were applied to the intermaxillary suture after force application into the intermaxillary suture of rats. Bone formation in the sutural area was histomorphometrically evaluated, including the amount of new bone formation (\(\text{mm}^2\)), number of osteoblasts, number of osteoclasts, and number of vessels. Mann-Whitney U-test was used for statistical evaluation at the \(P < .05\) level.

Results: After 10 days of retention, Pkh67\(^+\) can be detected in suture mostly in the injection site under fluorescence microscope. Histomorphometric analysis revealed that a single local injection of MSCs into the midpalatal suture increased the new bone formation in the suture by increasing the number of osteoblasts and new vessel formation, compared with controls injected with phosphate-buffered saline.

Conclusions: This preclinical study might provide foundations for the underlying potential clinical use of MSCs after maxillary expansion. Given the fact that MSCs are currently in use in clinical trials, this approach might be a feasible treatment strategy to accelerate new bone tissue formation in midpalatal suture and to shorten the treatment period for patients undergoing maxillary expansion reinforcement (Angle Orthod. 2015;85:394–399.)

KEY WORDS: Maxillary expansion; Bone regeneration; Bone marrow–derived stem cells; Orthodontics

INTRODUCTION

Shortening the healing period after maxillary expansion is crucial to achieve an effective treatment. Sawada and Shimizu\(^1\) applied a single dose of transforming growth factor–\(\beta\) 1 (TGF–\(\beta\)1) for stimulation of osteogenesis in expanding suture in rats. Various methods have been used to stimulate the deposition of new bone in an expanded area.\(^2,3\) However, the mechanism and stimulation of stress-mediated osteogenesis in the expanded suture by external factors are unclear.

Isolation and culture of MSCs are relatively easy compared with embryonic stem cells.\(^4\) MSCs are found in bone marrow, muscle, liver, placenta, peripheral blood, synovial fluid, dental pulp, and adipose tissue.\(^5\) They have been shown to differentiate into bone, cartilage, adipose, muscle, and nerve cells when...
stimulated with appropriate biological and chemical signals. MSCs originating from the bone marrow stroma are a particularly attractive source for osteogenic precursors for bone tissue engineering. It has been shown that local bone defects can be healed by site-directed transplantation of MSCs mostly with a scaffold made of biomaterials allowing for maximal MSC attachment, proliferation, and osteogenic differentiation and formation of mineralized matrix deposition. Not only bone defects but also local delivery of MSCs have been shown to increase wound healing, vascularization, and cartilage formation.

Bone marrow–derived MSCs (BMSCs) were shown to increase periodontal regeneration. Dental stem cells (DSCs), such as dental pulp stem cells and periodontal ligament stem cells, were also used in regeneration of dental tissues, including alveolar bone. Although DSCs are promising for regenerative dentistry, their availability is limited compared with BMSCs. Studies on the evaluation of the BMSCs on intermaxillary suture after expansion procedure were not found in the reviewed literature. In this study, we aim to show the effect of local MSC delivery on bone formation in response to expansion of the intermaxillary suture in a rat model.

MATERIALS AND METHODS

Ethical permission was obtained from the Erciyes University Ethics Committee of Experimental Animal Use and the Research Scientific Committee at the same institution for present study.

Cell Culture

Rat BMSCs were isolated and cultured as previously described. MSCs were obtained from six femora of syngeneic male Wistar rats. Briefly, after removal in aseptic conditions, the ends of the femoral bones were cut, and a 21-gauge needle was inserted followed by flushing of bone marrow with growth medium (Dulbecco's modified essential medium [DMEM] supplemented with 10% fetal bovine serum [FBS], 2 mM of L-glutamine, and 1% of penicillin, streptomycin, and fungizone). Bone marrow was pipetted vigorously for 5 minutes in a 50-mL sterile Falcon tube and centrifuged at 1500 rpm for 10 minutes. The cell pellets were resuspended in growth medium and cultured in T-150 flasks, with medium changed every other day until the attaching cell concentration reached 70%. Established cell lines were cultured in growth medium and incubated at 37°C in a humidified atmosphere of 5% CO₂ in the incubator. The cells were subcultured using trypsin-EDTA solution (1×). All cell culture reagents were purchased from Invitrogen (Carlsbad, Calif).

Flow Cytometry Analysis

The surface antigens of BMSCs were analyzed using flow cytometry analysis as previously described. Briefly, the cells at passage number 3 were trypsinized and incubated in phosphate-buffered saline (PBS) at 4°C for 45–60 minutes with primary antibodies, cluster differentiation (CD) 29 (cat No. SC-8978), CD90 (cat No. SC-9163), and CD45 (cat No. SC-25590; Santa Cruz Biotechnology Inc, Santa Cruz, Calif). After washing the excess primary antibodies, the cells were incubated with fluorescein isothiocyanate–conjugated secondary antibodies (cat No. SC-2012; Santa Cruz Biotechnology Inc) at 4°C for 45 minutes followed by washing with PBS and analyzing with flow cytometry. The flow cytometry analysis was carried out using the Becton Dickinson FACSCalibur flow cytometry system (Becton Dickinson, San Jose, CA).

Labeling MSCs With Green Fluorescent Protein and Osteogenic Differentiation of MSCs

All MSCs at passage 3 were labeled with cell membrane dyes PKH67 green fluorescent cell linker kit (cat No. MINI67, Sigma Aldrich, St Louis, Mo). Phk67- MSCs were used for experimental group.

To confirm MSC characteristics, the cells were differentiated into osteogenic cells based on protocols described previously. MSCs at passage 3 were used for differentiation experiments. Briefly, the cells were counted and cultured in six-well plates at a concentration of 3000 cell/cm² in growth medium. After 48 hours, growth medium was replaced with osteogenic medium: DMEM supplemented with 10% FBS (Invitrogen), 0.1 mM dexamethasone, 10 mM β-glycerol-phosphate, 50 mM ascorbate (Sigma Chemical Co, St Louis, Mo). Cells were incubated in osteogenic medium for 10 days, and the medium was changed every other day. On day 10, von Kossa staining and immunocytochemistry were performed to confirm osteogenic differentiation, as previously described.

Immunocytochemistry Analysis

After osteogenic differentiation, MSCs were fixed with 2% of p-formaldehyde and permeabilized by incubating with 0.1% Triton-X100/PBS for 5 minutes. Nonspecific binding of antibodies was blocked by adding 2% goat serum (diluted in PBS) for 20 minutes. Samples were incubated with primary antibodies (IgG, anti-collagen type-I (COL1A1; Santa Cruz Biotechnology, sc-59772) and anti-osteocalcin (OC; Santa Cruz Biotechnology, sc-30044), overnight at 4°C. Each sample was washed three times for 5 minutes with PBS to remove unbound primary antibodies. After washing, goat polyclonal anti-rabbit IgG-Alexa 488
conjugate (Invitrogen) secondary antibodies were added and incubated for 1 hour followed by washing three times. As a nuclear counterstain, DAPI (4',6-diamidino-2-phenylindole) was used (Sigma Chemical Co). Samples were observed under a Leica TCS SP2 SE confocal microscope (Leica, Bensheim, Germany) immediately after preparation.

von Kossa Staining

After 10 days of osteogenic differentiation in six-well plates, the cells were fixed with 2% of p-formaldehyde at 4°C for 30 minutes. After fixation, the cells were stained with von Kossa method (Bio-optica, Milano, Italy) following the kit instructions. Dark brown–stained calcium depositions were observed and pictured with a light microscope (Nikon TS100, Minneapolis, Minn).

Animals and Appliance Placement

Nineteen 50- to 60-day-old Wistar male rats with a mean weight of 241.67 ± 25.03 g were used. All animals were housed in polycarbonate cages, subjected to a 12-hour light-dark cycle at the constant temperature of 23°C, and fed a standard pellet diet (expanded pellets, Stepfield, Witham, Essex, UK) with tap water ad libitum.

The animals were anesthetized with an intramuscular injection of xylazine and xylasine. The expansion appliances were helical springs fabricated from 0.014-inch stainless-steel wire inserted in holes drilled close to the gingival margins of both upper incisors (Figure 1a). The springs were activated to deliver a force of 50 cN and were not reactivated during the 5-day expansion period. After 5 days, the springs were removed and replaced with short lengths of rectangular retaining wire.

Cell Delivery Into the Interpremaxillary Suture

Twenty-four hours after expansion started, the animals were anesthetized. The treatment group was injected with 1 × 10⁶ MSCs in 100 µL sterile saline, and the control group was injected with 100 µL sterile saline into the interpremaxillary sutures of animals (Figure 1b). All animals were weighed during the treatment period.

Specimen Preparation

After the retention period of 10 days, the rats were sacrificed with an overdose of ketamine and xylasine. The premaxillae were dissected out and fixed in 10% formalin. One of the premaxillae in the experimental group was randomly chosen and kept under −80°C. The sections were cut into blocks and sectioned serially at 10-μm intervals at −20°C with cryostat. The sections were counter-stained with DAPI and examined using fluorescent microscope under 4× and 40× objective (Figure 2).

Another 18 premaxillae were decalcified with 5% formic acid for 4 days. After decalcification, the premaxillae were cut into blocks, with one cut passing through the incisor crowns at the alveolar crest and perpendicular to the sagittal plane and the second cut 4 mm apical to the first cut. The sections were rinsed, trimmed, and embedded in paraffin. The paraffin blocks were sectioned serially at 5-μm intervals.

Histomorphometric Analysis

The histologic sections were stained with hematoxylin and eosin. The histomorphometric measurements were performed 200 μm beneath the oral surface of the osseous palate. Stained specimens were observed under a light microscope (Nikon EclipseE400, Nikon, Tokyo, Japan). For each specimen, the same area was pictured after staining by using a camera. The pictures were then transferred to a PC environment and calibrated with a Nikon Micrometer Slide. All photographs were evaluated with an image analysis program (Clemex Vision Lite Image Analysis3.5; Clemex Technologies, Longueuil, Canada). An area of 1.5 mm² in the center of the expanded suture was designated for both the experimental and control group. The amounts of osteoblasts, osteoclasts, and vessels in the newly formed bone region were marked in this area. The marked cells were counted automatically with the image.
analysis program. The amount of new bone formation ($\mu m^2$) also was measured (Figure 3).

**Statistical Analysis**

Results are expressed as mean ± standard deviation. Mann-Whitney $U$-test was performed where applicable for statistical calculations. Values of $P < .05$ were accepted as statistically significant.

**RESULTS**

MSCs were successfully isolated from bone marrow of rats and cultured until use. Flow cytometry data revealed that MSCs were positive for MSC markers CD29 and CD90 but negative for hematopoietic markers CD45 (Figure 4a). Cultured cells showed classical polygonal MSCs-like morphology (Figure 4b). Upon osteogenic differentiation, MSCs were stained positive with osteogenic markers COL1A1 (Figure 4c) and osteocalcin (Figure 4d), and then dark brown calcium depositions were visualized by von Kossa staining (Figure 4e).

All animals survived to the end of the study. The appliances were well tolerated, and the animals gained weight without significant changes during the treatment periods (Table 1).

Our histomorphometric analysis revealed that the group injected with MSCs had higher new bone formation than the PBS-injected group ($P = .035; 1.21$-fold). In correlation with this, we have found that the number of osteoblasts ($P < .043; 1.13$-fold) and vessels ($P = .016; 1.42$-fold) was higher in the MSC-treated group. On the other hand, we did not find a significant difference in the number of osteoclasts ($P = .288$) between the two groups (Table 2).

**DISCUSSION**

Following the maxillary expansion, the orthodontic treatment period is usually a hard time for the patients since having to wear an apparatus during the treatment is esthetically unpleasant and also causes mouth discomfort. Moreover, to prevent early relapse after expansion, it is crucial to induce new bone formation and regeneration. To achieve this goal, various therapeutic approaches were tried aiming to promote osteoblasts and thus improve the bone formation in the suture. Bringing limited success, these studies mostly included injection of bioactive molecules usually with a short half-life such as TGF-$\beta_1$ or vitamin D.

This study demonstrated that rat BMSCs express immunophenotypic characteristics similar to human

![Figure 3. Photomicrograph of a section in the expansion area of the experimental (n = 9) and control (n = 9) group (scale bar: 100 $\mu m$).](image1)

![Figure 4. Characterization and differentiation of MSCs. (a) Flow cytometry analysis shows that MSCs are expressing MSC markers CD29 and CD90, but they are not expressing hematopoietic marker CD45. (b) Classical polygonal shape of MSCs in cell culture. (c) Collagen type 1 staining of MSCs after differentiation. (d) Osteocalcin staining of MSCs after differentiation. (e) Dark brown calcium depositions visualized by von Kossa staining after osteogenic differentiation.](image2)

**Table 1. Body Weight Change (g) During the Study**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>T0 Mean</th>
<th>SD</th>
<th>T1 Mean</th>
<th>SD</th>
<th>T2 Mean</th>
<th>SD</th>
<th>T1–T0 Significance</th>
<th>T2–T0 Significance</th>
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<tbody>
<tr>
<td>Mesenchymal stem cells</td>
<td>10</td>
<td>249.22</td>
<td>23.26</td>
<td>237.89</td>
<td>25.77</td>
<td>237.89</td>
<td>18.42</td>
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<td>NS</td>
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<tr>
<td>Control</td>
<td>9</td>
<td>234.11</td>
<td>25.72</td>
<td>223.89</td>
<td>18.29</td>
<td>221.67</td>
<td>17.05</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* NS indicates not significant; SD, standard deviation; T0, start of experiment; T1, end of expansion (fifth day); and T2, end of retention (15th day).
MSCs and can differentiate along the osteogenic lineages. Based on flow cytometry analysis, rat MSCs were positive for CD29 and CD90 and negative for CD45, which is similar to human MSCs.6

Autologous MSCs have been investigated for the potential to regenerate a variety of tissues,20,21 and studies also have confirmed that the use of allogeneic MSCs can efficiently repair bone and other tissue types in different animal models without provoking an adverse immune response.14,22

In this study, we aimed to increase bone regeneration in the interpremaxillary suture using allogeneic BMSCs. This strategy has two advantages. The first advantage is that MSCs derived from bone marrow are able to differentiate into osteogenic cells. Therefore, upon being stimulated by microenvironmental factors, they are involved in new bone formation at the site of injection. The second advantage of MSCs over other methods is that MSCs can tune up new bone formation by secreting growth factors such as bone morphogenetic protein 2 or vascular endothelial growth factor, which are key players in new bone formation.18

In the current study, the newly formed osteoblasts, osteoclasts, and vessels in the interpremaxillary suture region in the MSC-treated group were identified through histomorphometric assessment. The data revealed that new bone formation was higher in the MSC-treated group compared with the control group. A single local injection of MSCs enabled faster bone formation following the maxillary expansion by increasing the number of osteoblasts and vessels in the midpalatal suture region. DAPI is a fluorescent stain that binds strongly to A-T–rich regions in DNA. DAPI can pass through an intact cell membrane; therefore, it can be used to stain both live and fixed cells, although it passes through the membrane less efficiently in live cells and therefore the effectiveness of the stain is lower. It is used extensively in fluorescence microscopy.

We have used Phk67+ MSCs and DAPI staining for the presence of MSCs in the sutural area as distinct from previous studies23,24 evaluating bone healing in animal models regarding the application of MSCs. Phk67+ MSCs were detected in newly formed bone at 2 weeks. We proved that applied stem cells have participated to the new bone structure.

Today, BMSCs are clinically available autologous stem cell sources for humans.21 Clinical applications of this approach might reduce the relapse rate after treatment and achieve more stable results. Since a shorter treatment period for patients undergoing the maxillary expansion treatment will ensure that the patients will not have to use an apparatus for a long period of time, it might result in a high level of patient cooperation, improving the comfort of both patients and physicians. However, before clinical applications, further studies are needed with larger and more complex animal models that have an anatomy closer to that of the human anatomy.

CONCLUSIONS

• In this study, BMSCs were isolated from the femora of rats, and characterization of MSCs and osteogenic differentiation was performed. Positive contribution to bone formation was detected with differentiated MSCs that underwent a successful stem cell therapy in the maxillary expansion model in rats.
• Histomorphometric findings showed an increase in newly formed bone, number of osteoblasts, and amount of vascularization with locally applied MSCs to the expanded maxilla.
• It was shown via a fluorescent microscope that Phk67+ MSCs participated in bone structure.

REFERENCES


Table 2. Descriptive Values and Mann-Whitney U-test Comparisons of Histomorphometric Measurementsa

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Minimum</th>
<th>Maximum</th>
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<tr>
<td>New bone formation area, µm²</td>
<td>Control</td>
<td>9</td>
<td></td>
<td>395078.6</td>
<td>90610.3</td>
<td>240644.5</td>
<td>499967.5</td>
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<tr>
<td></td>
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<td>418458.32</td>
<td>28416.95</td>
<td>390009</td>
<td>470665.2</td>
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<tr>
<td>Number of osteoblasts</td>
<td>Control</td>
<td>9</td>
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<td>12.81</td>
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<tr>
<td></td>
<td>BMSC</td>
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<td>65.78</td>
<td>11.57</td>
<td>49</td>
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<td></td>
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<tr>
<td>Number of osteoclasts</td>
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<td></td>
<td>5.8</td>
<td>1.07</td>
<td>3.0</td>
<td>8.0</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>BMSC</td>
<td>9</td>
<td></td>
<td>4.56</td>
<td>3.26</td>
<td>2.0</td>
<td>11.0</td>
<td></td>
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<tr>
<td>Number of vessels</td>
<td>Control</td>
<td>9</td>
<td></td>
<td>5.0</td>
<td>1.64</td>
<td>3.0</td>
<td>9.0</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>BMSC</td>
<td>9</td>
<td></td>
<td>8.56</td>
<td>8.18</td>
<td>2</td>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>

* SD indicates standard deviation; BMSC, bone marrow–derived mesenchymal stem cell; and NS, not significant.
* P < .05.
Erratum

Bone marrow mesenchymal stem cells enhance bone formation in orthodontically expanded maxillae in rats by Abdullah Ekizer, Mehmet Emir Yalvac, Tancan Uysal, Mehmet Fatih Sonmez, Fikrettin Sahin

The Angle Orthodontist, 2015;Vol. 85, No. 3 pp. 394–399

The 3rd paragraph of the RESULTS of this paper should be replaced with the following:

Our histomorphometric analysis revealed that the group injected with MSCs had higher new bone formation than the PBS-injected group ($P < .05$; 1.06-fold). In correlation with this, we have found that the number of osteoblasts ($P < .05$; 1.2-fold) and vessels ($P < .05$; 1.71-fold) was higher in the MSC-treated group. On the other hand, we did not find a significant difference in the number of osteoclasts ($P = .288$) between the two groups (Table 2).

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