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**Regenerative Techniques in Alveolar Cleft Osteoplasty:
Concomitant use of Buccal fat pad derived cells and
autogenous bone in a preliminary study**

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Regenerative Techniques in Alveolar Cleft Osteoplasty: Concomitant use of Buccal fat pad derived cells and autogenous bone in a preliminary study

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ABSTRACT

Recently, tissue regeneration has become a promising treatment for bone defects, eliminating donor site morbidity. There are few applications of tissue engineering in alveolar cleft defects. The purpose of this study was to compare the outcomes of intra- and extra-oral autogenous bone grafts and buccal fat pad (BFP)-derived mesenchymal stem cells (MSCs) using the gold standard method to repair human alveolar cleft bone defects. Ten patients with unilateral cleft lip and palate were selected for this study, and anterior maxillary cleft defects were treated based on group assignment. Group 1 was treated with anterior iliac crest (AIC) bone and a collagen membrane, Group 2 was treated with lateral ramus cortical bone (LRCP) with MSCs mounted on a freeze-dried bone allograft, and Group 3 was treated with AIC bone, MSCs cultured on natural bovine bone mineral, and a collagen membrane. The amount of regenerated bone was measured using cone beam computed tomography 6 months post-operatively. Greater amounts of new bone were formed in Group 3, suggesting that use of BFP-derived MSCs with AIC bone can increase bone regeneration capacity in alveolar cleft bone defects; however, the differences were not statistically significant.

Keywords: Alveolar cleft; Osteoplasty; Tissue engineering; stem cells; regenerative medicine

1. Introduction

The most common facial congenital malformation, cleft lip and palate, can disturb patient functions and cause psychological problems (*Luaces-Rey et al.*, 2010; *Bueno et al.*, 2011; *Yoshioka et al.*, 2012). Fusion failure of the nasal process and oropalatal shelves leads to alveolar cleft in 0.36 to 0.83 out of every 1000 live births (*Pradel et al.*, 2008; *Vecchiattini et al.*, 2009; *Luaces-Rey et al.*, 2010). Seventy-five percent of all cleft lip and palate variations are accompanied by alveolar bone defects (*Waite and Waite*, 1996; *Guo et al.*, 2011).

Secondary alveolar bone grafting is necessary for maxillary arch integration, easier teeth eruption, alar base support, and closure of oroantral communication (*Luaces-Rey et al.*, 2010; *Behnia et al.*, 2012). The iliac crest is the most popular donor site for harvesting autogenous bone (*Behnia et al.*, 2012). Hospitalization time, pain, donor site morbidities, cost, and late bone resorption are the primary disadvantages of alveolar cleft autografting (*Gimbel et al.*, 2007; *Behnia et al.*, 2012). Tissue engineering merges the fields of cell biology, biomaterial engineering, and medicine to fabricate new functional tissues (*Pradel et al.*, 2008; *Luaces-Rey et al.*, 2010; *Behnia et al.*, 2012). It reduces donor site morbidity, postoperative pain, inadequate bone regeneration, additional cost, and hospitalization (*Herford et al.*, 2007; *Behnia et al.*, 2009; *Le and Woo*, 2009; *Lee et al.*, 2009). Growth factors and mesenchymal stem cells (MSCs) are two major approaches for bringing tissue engineering from the bench to the bedside (*Pradel et al.*, 2008; *Behnia et al.*, 2012). The most commonly-used growth factor in bone engineering, RhBMP-2, resulted in 65% to 95% bone fill in cleft defects (*Dickinson et al.*, 2008; *Alonso et al.*, 2010). Mesenchymal stem cells are multipotential cells that can be harvested from mesodermal tissue such as bone marrow, dental pulp, periostium, and fat (*Khojasteh et al.*, 2012; *Morad et al.*, 2013). They effected 25% to

79% new bone formation in alveolar cleft defects (*Hibi et al.*, 2009). A combination of MSCs and platelet-derived growth factors demonstrated 51% new bone in secondary alveoloplasty (*Behnia et al.*, 2012). A wide range of data and lack of predictability has led to a continuing search for better results (*Dickinson et al.*, 2008; *Behnia et al.*, 2009; *Alonso et al.*, 2010). Cell sources, differentiation protocols, incubation periods, passage counts, types of scaffolds, and delivery methods varied between studies (*Khojasteh et al.*, 2013a). Our aim was to combine regenerative techniques with bone grafting in human models to increase predictability and survival of reconstructed tissue. The MSCs in this study were used with bone grafts that were harvested from either an intra-oral donor site or an extra-oral donor site. Results were compared.

2. Materials and methods

2.1. Patients

Ten patients (3 female) with unilateral cleft lip and palate were selected for this study. The only remaining surgical procedure in each patient was secondary alveolar bone grafting. Four patients were adults, and therefore had lost the chance for canine eruption. All others were aged 8 to 14 years and had concomitant growing teeth in the cleft space. All patients received presurgical orthodontic treatment. Computed tomography was used to assess defect location and size. Permission was obtained to complete the procedure despite the absence of well-documented data supporting stem cell-induced bone regeneration in cleft patients. All procedures were approved by the institutional ethical committee, and informed consent was obtained from all donors. Isolation and cultivation of the MSCs was performed without xenogenic supplements such as fetal calf serum (FCS). All methods used to cultivate and extract MSCs was as described in previous projects using human patients (*Shayesteh et al.*, 2008; *Behnia et al.*, 2009; *Behnia et al.*, 2012).

Patients were divided into 3 groups. Group 1 (3 patients), the control group, was treated with anterior illiac crest (AIC) spongy bone to fill defects, followed by coverage with collagen membrane. Group 2 (3 patients) was treated with lateral ramus cortical bone (LRCP), used to create a protected healing space by fixing it to adjacent walls of the cleft defect. Buccal fat pad (BFP)-derived MCSs were loaded onto a freeze-dried bone allograft (FDBA) and delivered to the defect. To harvest an LRCP of the appropriate size, the patient should have a mature ramus with erupted molars. Adult patients were selected for this group. Group 3 (4 patients) was treated with (AIC) as in Group 1, but BFP-derived MSCs cultured over natural bovine bone mineral (NBBM) were put over the spongy bone and covered with a collagen membrane.

2.2. Isolation of MSCs from the BFP

The BFP was harvested from healthy donors through a vestibular incision distal to the maxillary second molar (Fig. 1). It was exposed with blunt dissection while preserving the thin covering membrane. About 3 to 5 ml were excised and delivered to the lab in DMEM medium. Dissected tissue was minced and incubated in 3 mg/ml type I collagenase and 4 mg/ml dispase in phosphate buffered saline (PBS) (GIBCO Laboratories, Grand Island, NY, USA) at 37 °C for 30 min. The fragments were then immersed in α -MEM culture medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (GIBCO) and placed in 25-cm² culture flasks followed by incubation in a humidified atmosphere containing 5% CO₂ at 37 °C. Culture media was changed twice weekly and after reaching the subconfluent stage, and cells were removed by enzymatic digestion (0.25% trypsin–EDTA) and passaged. MSCs of the third to fourth passage were subjected to the experiments. Adherent cells were expanded as monolayer cultures in a 95/5 air/CO₂ (v/v) atmosphere at 37 °C with media changes every 3 days. Confluent cells were dissociated with trypsin and subcultured in new 6-well culture dishes at a plating density of 6×10^4 cells/dish. The cells could be seen under light microscopy (Fig.2).

2.3. Preparation of human serum

Fetal calf serum was replaced by human serum because of concerns of the ethical committee. From each patient, 20 ml whole blood was drained into blood bags (Baxter, Deerfield, IL), quickly transferred to 10-ml Vacutainer® tubes without anticoagulants (BD, Plymouth, UK), and allowed to clot for 4 h at 4 to 8 °C. Subsequently, the blood was centrifuged at 1800 g at 4 °C for 15 min. Serum was collected and filtered through a 0.2-mm membrane (Sarstedt, Nümbrecht, Germany). Aliquots of the sterile serum

were stored at -20 °C. The lab process and cultivation of cells from each patient was performed without significant salience.

2.4. Evaluation of the MSC nature of the isolated cells

2.4.1. Flow cytometry analysis

Fluorescence absorbance cell sorting (FACS) analysis was performed using standard protocols and quantification criteria to confirm cell surface expression of CD44, 90, 73, 105 and 45. The gate to distinguish positive from negative cells was set individually for each marker. Fluorescent isothiocyanate (FITC)-conjugated monoclonal antibodies were applied to the isolated cells using the flow cytometry device (BD FACS Calibur, Franklin Lakes, NJ). anti-CD44-FITC, anti-CD90-FITC, anti-CD73-PE, anti-105-PE, anti-CD45-FITC and anti-CD34-PE (EXBIO, Vestec, Czech Republic).were used at 2 µg/ml. Cells stained with FITC-labeled mouse IgG were used as negative controls. After being washed twice with PBS, cells were fixed with 1% paraformaldehyde. Flow cytometry standard files analysed by flowjo 7.6.1 software. Specimens containing at least 90% fluorescent-labeled cells were regarded as positive.

2.4.2. Differential potential

The differentiation potential toward osteogenic and adipogenic lineage were examined for the stemness of the cultured cells. Third passage-cultured cells were treated with osteogenic medium containing 10 mmol β-glycerol phosphate (Sigma-Aldrich, St. Louis, MO or Taufkirchen, Germany), 50 µg/ml ascorbic-2-phosphates (Sigma), and 10⁻⁷ M dexamethasone (Sigma) for 3 weeks. Subsequently, to verify osteogenic differentiation, stem cells were fixed with 4% paraformaldehyde for 10 min and stained with alizarin red. Adipogenic medium containing DMEM with 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, St. Louis, MO or Taufkirchen, Germany), 250 nM dexamethasone, and 0.2 mM indomethacin was also used to induce adipogenic

differentiation of third passage cells. Cells were fixed using 4% paraformaldehyde, washed with 70% ethanol, and stained using oil red solution in 99% isopropanol for 15 min.

2.5. Implant preparation

In all cases, 7 days before transplantation, implants were loaded with the cells obtained from the third subculture. SureOss (HansGBR Biomaterial, Seoul, Korea) is a freeze-dried cortical bone allograft; a granular biomaterial with a 200- to 850- μ m particle size.

2.6. Scanning electron microscopic analysis

Cell-loaded scaffolds were fixed in 2.5% glutaraldehyde (Merck KGaA, Darmstadt, Germany), washed with PBS, dehydrated in graded ethanol, vacuum dried, and coated with gold. The prepared block was examined under a scanning electron microscope (Hitachi, Tokyo, Japan).

2.7. Surgical procedure

Surgery was performed under general anesthesia. Following a crestal incision at the level of the gingival sulcus, dissections were made in the scar tissue to reach the bony surface of the cleft walls. The tissue was then elevated beneath the periosteum plane to the level of the anterior nasal spine anteriorly, the lateral piriform rim superiorly, and the alveolar ridges inferiorly. The flaps of the nasal floor and the oral mucosa formed the ceiling and floor of the cleft cavity, respectively. Concomitant injection of Cefazolin (1 g) was used during the perioperative period and followed by a 3-day course. In Group 2, LRCP was harvested from one or both sides of the mandibular ramus according to the defect size (Fig. 3). Lateral ramus cortical bone was trimmed and split into 2-3 pieces to create a protected healing space in the alveolar cleft defect. The mixture of scaffolding and cells was transferred to the defect using microforceps (Fig. 4

A-E). The wound was subsequently closed in a water-tight tension-free manner. In Groups 1 and 3, cleft defects were treated with AIC spongy bone by placing the cortical bone in the nasal floor and covering with a collagen membrane (Fig 5 A-C). In Group 3, cell-loaded FDBA was put over the AIC spongy bone and covered with collagen membrane (Fig 6 A-D). Use the "Insert Citation" button to add citations to this document.

2.8. Clinical evaluation

Soft tissue healing and normal healing sequences of grafted tissues were evaluated every 2 weeks.

2.9. Radiographic evaluation

Cone beam computed tomography was obtained after 6 months. The outline of 1-mm coronal sections of the treated region were taken before and after surgery, and new bone formation was assessed. The outlined sections of the alveolar defect were used to determine the preoperative defect, postoperative defect, and volume of bone fill using Image Pro software (National Institutes of Health [NIH], Bethesda, MD).

2.10. Data analysis

All statistical analyses were performed using a software package (SPSS Statistics 18; SPSS Inc., Chicago, IL, USA). A significance level of 0.05 was used for all comparisons.

3. Results

3.1. In vitro differentiation

3.1.1. Flow cytometry

In order to confirm whether the isolated/cultured BFPSCs display the mesenchymal stem cell (MSC) phenotype, cells were subjected to flow cytometry analysis for expression of positive CD44, CD90, CD73 and CD105 and negative (CD45 and CD34) MSC markers. The result showed that more than 95% of this cell population were positive for CD44, CD90, CD73 and CD105, MSC-surface markers, whereas they were negative for CD45 and CD34, hematopoietic markers (Fig. 7).

3.1.2. Cell differentiation

Cells have the ability to differentiate into either osteogenic or adipogenic lineage as MSCs, and this was observed using inverted light microscopy at 3 weeks postculture. Nodule-like structures of mineralized matrix were detected in the former, whereas the latter demonstrated lipid droplets within cells' cytoplasm, proving the multipotency of the isolated cells (Fig. 8 A,B).

3.2. Scanning electron microscopy

Scanning electron micrographs showed MSCs were scattered within the pores of the scaffold. Adherence to the scaffold was demonstrated by cellular pods and attachments (Fig. 9 A,B).

3.3. In vivo bone formation

Successful healing with no fistula or oronasal communication was achieved in all cases except for one patient in Group 2. This patient experienced partial palatal fistula, had the least amount of bone formation (10%), and was omitted from the study. In the radiomorphometric data. Radiomorphometric values for new bone formation in cleft defects are shown in Table 1. After 6 months, members of Group 2 (LRCP +MSCs)

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experienced between 65% and 85% new BF (Fig. 10) while for those in Group 3 (AIC +MSCs), it was 70%, 85%, and 90% (Fig. 11). The controls (AIC) experienced 65%, 70%, and 85% new bone (Fig. 12). The mean new bone formation was highest in Group 3, but not at a statistically significant level ($p = 0.054$; Table 2).

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4. Discussion

Application of regenerative techniques, either through cells or growth factors, to enhance the healing capacity of the body or decrease donor site morbidities, has been a gateway for bringing tissue engineering principles from the bench to the bedside. Mesenchymal stem cells derived from human bone marrow appear to be a popular source of adult stem cells and are now widely used in various cell therapies and tissue engineering procedures (*Khojasteh et al.*, 2012). Using craniofacial sources such as dental pulp or BFPs, a better niche for regenerative techniques in oral and maxillofacial areas may be provided (*Morad et al.*, 2013).

Use of MSCs in various kinds of animal models has been successful in forming new bone (*Eslami et al.*, 2008; *Jafarian et al.*, 2008; *Behnia et al.*, 2013; *Khojasteh et al.*, 2013b). Applications of these stromal cells, with or without adhesive agents, in some critically-sized defects demonstrated less than optimal bone formation to heal the defect completely (*Khojasteh et al.*, 2013c; *Khojasteh et al.*, 2014).

Using MSCs for maxillary sinus augmentation has also shown acceptable results (*Shayesteh et al.*, 2008; *Kaigler et al.*, 2015). Maxillary alveolar cleft defects behave like critically-sized defects in the healing sequence. Using autologous bone grafts, alveolar cleft osteoplasty is the standard treatment for the management of alveolar clefts (*Rychlik and Wojcicki*, 2012).

Pradel et al. demonstrated the successful use of differentiated osteogenic cells for cleft repair in a case report, concluding that the method can lead to spontaneous tooth eruption on the cleft side (*Pradel et al.*, 2008).

In previous experiments, bone marrow MSCs, when cultured over a composite scaffold of demineralized bone and calcium sulfate showed 24% new bone formation in alveolar cleft defects (*Behnia et al.*, 2009). Using MSCs with the same technique of harvesting

and delivery, when cultured over biphasic hydroxy apatite/tricalcium phosphate and mixed with the platelet rich in growth factor (PRGF) as an adhesive agent, resulted in increased bone formation of up to 50% (*Behnia et al.*, 2012).

Lateral ramus cortical bone is one of the most common intra-oral donor sites used to treat atrophic ridge in reconstructive surgery (*Khojasteh et al.*, 2011; *Khojasteh et al.*, 2013d). In this study, it was used in 3 patients to create a protected healing space fixed between the nasal floor and the buccal and palatal side of the cleft defect. Using LRCP as an autogenous barrier containing MSCs increased the rate of new bone formation and resulted in 65% to 85% BF. In one member of Group 2, a partial palatal fistula occurred, and BF decreased to 10%. All members of this group were adults (aged 20 to 29 years) and had lower regenerative capacities.

We also loaded MSCs into scaffolds over AIC bone as a barrier to prevent secondary bone resorption. In this group, the range of BF was between 75% and 90%, higher than that seen using AIC alone (controls), where it was 65% to 85%. Although tissue-engineered bone did not show a high level of evidence for treatment of critically-sized defects (*Khojasteh et al.*, 2012), applying regenerative techniques with autogenous donor sites seemed to enhance bone formation capacity in the alveolar cleft defects and can decrease the amount of bone needed from harvesting. This concept can decrease donor site morbidities and time of hospitalization.

Conclusion

Combination of MSCs with AIC bone can enhance new bone formation in alveolar cleft bony defects. Intra-oral donor sites such as LRCP may be used as a cage to protect scaffolds loaded with MSCs.

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Tables

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Table 1. Clinical and radiomorphometric amounts of new regenerated bone; abbreviations: OTM, orthodontic tooth movement; F, female; M, male; BF, bone formation; BFP, buccal fat pad, AIC, anterior iliac crest; MSCs, mesenchymal stem cells; LRB, lateral ramus bone.

Cases		Group	Age	Gender	OTM	SET	MSCs Source	Defect Volume	Result (soft tissue healing and new Bone formation) at 6 month
1	A	LRB+MSCs	21	F	+	—	BFP	6*10*15=5.5	- Complete closure - 30% BF at 4 month - 85% BF at 6 moth
2	A	LRB+MSCs	29	M	—	—	BFP	9.3 * 10.1 * 8.5	- Complete closure - 65% BF
3	A	LRB+MSC	28	M	+	—	BFP	10.3*11.1*9.2	- partial fistula in the palatal side - 10% BF
1	B	AIC+MSCs	14	M	+	—	BFP	11.6* 12.9* 7	- Complete closure - 85% BF
2	B	AIC+MSCs	10	M	+	+	BFP	13.4*5.2 * 9.1	- Complete closure - Normal eruption of adjacent teeth - 70 % BF
3	B	AIC +MSC	8	M	+	+	BFP	9.2* 12 *8.4	- Complete closure - Normal eruption of adjacent teeth - 90 % BF
4	B	AIC+MSCs	22	F	—	—	BFP	5.4*8.1*9.2	- Complete closure - 85% BF
1	C	AIC	17	M	+	—	BFP	12.2* 8.6 * 6.4	-65 % BFComplete closure
2	C	AIC	11	F	—	—	BFP	6.3 * 9.4 * 7.5	- Complete closure - 70 % BF
3	C	AIC	8	M	+	+	BFP	9.2* 12* 8.4	- Complete closure -Normal eruption of adjacent teeth - 85% BF

Table 2. Comparison of mean bone formation between groups; *10% bone formation happened in a case with partial fistula; abbreviations: Min, minimum; Max, maximum; MSCs, mesenchymal stem cells; LRCP, lateral ramus cortical bone.

Group	Number	Defect mm³ [Mean ± SD]	Bone Fill % [Mean ± SD]	Min (Bone Fill %)	Max (Bone Fill %)
1 (Iliac)	3	6.80 ± 2.41	70 ± 10.40	65	85
2(MSCs+LRCP)	3	7.99 ± 2.50	65 ± 3.5	10*	75
3(MSCs+Iliac)	4	7.52 ± 2.91	82.5± 6.45	75	90

FIGURES

Fig. 1. Buccal fat pad harvesting; Buccal fat pad was exposed and harvested using a vestibular incision distal to the second molar.

Fig.2. Light microscopic evaluation of the stellate like cells extracted from buccal fat pad.

Fig. 3. Lateral ramus cortical bone was harvested from lateral side of the mandible.

Fig. 4 (A-E). Preparation and placing of LRCP; LRCP was trimmed and cut to 2-3 pieces and fixed in defect region. Protected healing space was created and BFPCs were delivered to the space.

Fig.5 (A-C). Anterior iliac crest spongy bone was filled in the alveolar defect in control group and covered with collagen membrane.

Fig.6. (A-D). Scaffolds carrying BFPCs were covered the spongy iliac bone in AIC +MSCs group.

Fig. 7. Flowcytometric evaluation of the human buccal fat pad derived stem cells. CD 70, 93,44 and 105 were detected positive and CD 45 and 34 were negative.

Fig.8. A: Alizarin red staining; Nodule-like structures of mineralized matrix were observed under inverted light microscope; B: Oil red staining revealed positive result for invitro adipogenic differentiation.

Fig. 9. A: SEM evaluation views of FDBA granule (×50); B: BFPCs laid down into the scaffold through cellular pods and attachments. (×1000)

Fig. 10. Tomographic of new bone formation in lateral ramus protected group.

Fig. 11. Tomographic of new bone formation in group 2;

Fig. 12. Radiographic investigation of new bone formation in control group

For Peer Review

Buccal fat pad harvesting; Buccal fat pad was exposed and harvested using a vestibular incision distal to the second molar.
178x151mm (119 x 119 DPI)

Light microscopic evaluation of the stellate like cells extracted from buccal fat pad.
178x133mm (146 x 146 DPI)

Lateral ramus cortical bone was harvested from lateral side of the mandible.
178x107mm (139 x 139 DPI)

Preparation and placing of LRCP; LRCP was trimmed and cut to 2-3 pieces and fixed in defect region.
Protected healing space was created and BFPCs were delivered to the space.
299x261mm (72 x 72 DPI)

Preparation and placing of LRCP; LRCP was trimmed and cut to 2-3 pieces and fixed in defect region.
Protected healing space was created and BFPCs were delivered to the space.
325x267mm (72 x 72 DPI)

Preparation and placing of LRCP; LRCP was trimmed and cut to 2-3 pieces and fixed in defect region. Protected healing space was created and BFPCs were delivered to the space.
178x154mm (120 x 120 DPI)

Preparation and placing of LRCP; LRCP was trimmed and cut to 2-3 pieces and fixed in defect region. Protected healing space was created and BFPCs were delivered to the space.

178x161mm (112 x 112 DPI)

For Peer Review

Anterior iliac crest spongy bone was filled in the alveolar defect in control group and covered with collagen membrane.
178x127mm (136 x 136 DPI)

Anterior iliac crest spongy bone was filled in the alveolar defect in control group and covered with collagen membrane.
179x131mm (126 x 126 DPI)

Anterior iliac crest spongy bone was filled in the alveolar defect in control group and covered with collagen membrane.
178x122mm (118 x 118 DPI)

Anterior iliac crest spongy bone was filled in the alveolar defect in control group and covered with collagen membrane.

178x124mm (145 x 145 DPI)

Anterior iliac crest spongy bone was filled in the alveolar defect in control group and covered with collagen membrane.
178x123mm (131 x 131 DPI)

Anterior iliac crest spongy bone was filled in the alveolar defect in control group and covered with collagen membrane.
178x127mm (140 x 140 DPI)

Anterior iliac crest spongy bone was filled in the alveolar defect in control group and covered with collagen membrane.
178x141mm (129 x 129 DPI)

Flowcytometric evaluation of the human buccal fat pad derived stem cells. CD 70, 93,44 and 105 were detected positive and CD 45 and 34 were negative.
178x114mm (124 x 124 DPI)

Alizarin red staining; Nodule-like structures of mineralized matrix were observed under inverted light microscope
178x133mm (146 x 146 DPI)

Oil red staining revealed positive result for invitro adipogenic differentiation.
178x131mm (135 x 135 DPI)

A: SEM evaluation views of FDDBA granule (×50)
177x122mm (144 x 144 DPI)

BFPCs laid down into the scaffold through cellular pods and attachments. ($\times 1000$)
178x113mm (145 x 145 DPI)

Tomographic of new bone formation in lateral ramus protected group.
177x73mm (146 x 146 DPI)

Tomographic of new bone formation in group 2
177x70mm (141 x 141 DPI)

Radiographic investigation of new bone formation in control group
177x52mm (137 x 137 DPI)