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THE CLINICAL USE OF AUTOLOGOUS BONE MARROW MESENCHYMAL STEM CELLS IN THE TREATMENT OF SECONDARY ALVEOLAR CLEFT

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ABSTRACT

Objective: To compare the clinical outcome of alveolar cleft bone grafting with expanded mesenchymal stem cells (MSCs) seeded into a resorbable matrix to standard iliac cancellous bone graft. **Subjects and methods:** Fifteen patients with unilateral alveolar cleft aged 7-12 years old (eight girls and seven boys) were randomly divided, according to grafting technique, into two groups: Group I: Alveolar cleft grafting with expanded autogenous bone marrow stem cells seeded in collagen sponge, platelet rich plasma and nanohydroxyapatite. Group II: Alveolar cleft grafting with cancellous bone harvested from anterior iliac crest. Follow up clinical evaluation on the first postoperative day, every other day for the first week, weekly thereafter for a month then every month for six months; concerning postoperative pain, soft tissue healing, oronasal fistula closure and tooth eruption. **Results:** All patients in group I experienced no pain or discomfort at the bone marrow aspiration site while patients in group II experienced pain scored 3 by VAS and disappeared gradually within a week. Wound dehiscence represented 14% of group II. After six months postoperatively eruption of lateral incisor was observed in 25% in group I versus 14% in group II. **Conclusion:** The effect of stem cell tissue engineering did prove to have a positive clinical result compared to gold standard used in alveolar cleft grafting and provided significant support to the healing of soft tissues around the alveolar cleft. Tissue engineering bone graft is a cost effective and require two setting comparable to the autogenous bone graft.

KEYWORDS: Autologous Bone Marrow, Mesenchymal Stem Cells, Alveolar Cleft

INTRODUCTION

Alveolar cleft is a common congenital anomaly which affects approximately 75% of cleft lip and palate patients. The etiology of this cleft is still poorly understood, but it is most likely considered to be multifactorial involving genetic and environmental factors [(1)]. Alveolar bone grafting is an integral part of the treatment in patients with unilateral or bilateral cleft lips and palates [(2,3)]. The primary goal of alveolar cleft reconstruction is to provide a bony bridge at the cleft site that allows maxillary arch continuity, oronasal fistula repair, eruption of the permanent dentition into the newly formed bone and improvement of the periodontal conditions. Teeth that are well covered by bone can be manipulated orthodontically with less risk of root exposure and subsequent tooth loss. Other reasons

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for alveolar bone grafting are establishing better oral hygiene, and support for the alar base of the nose [4]. Autogenous bone was reported as the proper bone graft, because it supplies living bone cells essential for osteogenesis. Therefore, its transplantation is still the gold standard when harvested from sites such as the iliac crest, the mandible, the tibia and the cranium [5,6].

However, they are often related to disadvantages such as limited availability, donor site morbidity, possible hospitalization and the need for general anesthesia [7]. To overcome these limitations, several substitutional biomaterials such as hydroxyapatites, β-tricalcium phosphates, and demineralized bone matrices are in clinical use [8-10]. Their combination with stem cell and growth factors provides a new alternative where stem cells are seeded on 3-dimensional bone-like scaffolds [11,12].

Although The clinical application of stem cell-based tissue engineering is promising in preclinical and clinical studies [11,13-15]. There is a limitation in randomized controlled clinical trials that are needed to judge the efficiency of the stem cells in reconstruction of alveolar defect, a factor that initiated us to start the present study. We aimed to see whether the clinical outcome of tissue-engineered bone with stem cells would function like standard iliac cancellous bone in alveolar cleft reconstruction.

SUBJECTS AND METHODS

A prospective randomized controlled clinical study was approved by the ethical committee of faculty of dental medicine, Al-Azhar university. Fifteen patients with unilateral alveolar cleft with mean age of 9.93 years old (range: 7-12 years, eight girls and seven boys) were selected from those attending the outpatient clinic of Oral and Maxillofacial Surgery, Faculty of Dental Medicine, Al-Azhar University, Cairo, Egypt; according to the following inclusion criteria: patients with unilateral alveolar cleft that require secondary alveolar cleft grafting and aged from 7 to 12 years old. However, patients with bilateral alveolar cleft, patients with previous attempt of surgical closure of alveolar cleft, patients with diseases affecting bone healing i.e. patients on cortisone and diabetic patients, severely misaligned maxillary segment that require successive surgical and orthodontic correction before grafting and syndromic patients were excluded from the study. The parents signed a written consent form having all details about the procedure and acceptance to be a part of this study**.** All patients were randomly divided, according to grafting technique, into two groups**:**

Group I: Alveolar cleft grafting with expanded autogenous bone marrow stem cells seeded in collagen sponge, platelet rich plasma and nanohydroxyapatite.

Group II: Alveolar cleft grafting with cancellous bone harvested from anterior iliac crest.

I- Patient evaluation:

Patient history and clinical examination were recorded including soft tissue condition, gingival health, presence of labionasal and palatal fistulae and dental condition. Also, pre-operative CBCT was requested on maxilla for assessment of bone defect and dental status.

II- Surgical procedure:

A. Donor site preparation:

Cell Preparation (Group I, Stem cell group): Under deep sedation and local anesthesia, 20 ml bone marrow was aspirated in the operating theatre under aseptic conditions from anterior iliac crest with bone marrow aspiration needle and placed in heparinized tubes four weeks before grafting for culturing and expansion. The bone marrow aspirate was carried on ice in sealed containers to the Molecular Biology and Tissue Engineering Unit (MBTEU) in the Department of Medical Biochemistry at Cairo University where cell cultures were performed. Briefly, nucleated cells were isolated from the bone marrow aspirate with a density gradient [Ficoll/Paque (Pharmacia)] and re-suspended in complete culture medium

[Delbecco's Modified Eagle's Medium (DMEM) (GIBCO/BRL)] supplemented with 10% Fetal Bovine Serum (GIBCO/BRL) and 1% Penicillin-Streptomycin (10,000 μg/ml) (GIBCO/BRL). FBS used in this study was purchased from reliable countries without bovine virus traceability where it is furthermore subjected to serial filtration, terminal sterilization with gamma irradiation, followed by bacterial and viral testing using validated procedures to ensure pathogen-freedom. Cells were incubated at 37 °C in 5% humidified CO2 for 12–14 days after which the number of adherent cells increased and reached an average of 3 x 10^6 as counted by the hemocytometer. The culture medium was changed every two to three days. When large colonies developed (80–90% confluence), cultures were washed twice with phosphate buffer saline (PBS) and the cells were trypsinized with 0.25% Trypsin in 1mM EDTA (GIBCO/BRL) for 5 min at 37°C. After centrifugation, cells were re-suspended with serum-supplemented medium and incubated in 50 cm2 flasks (Falcon) and sub-cultured for approximately another 10 days, reaching an average count of 5 x 10^6. MSCs in culture were characterized by their adhesiveness and fusiform shape, by flow cytometry to confirm MSCs surface markers (CD34-, CD45- and CD73+) and by CD29 gene expression by RT-PCR as a marker of MSCs [16,17]. On the day of implantation, the sub-cultured cells were trypsinized, collected and resuspended in 1 ml PBS and transferred to the operating room in sterile tubes. Potential immunogenicity from FBS proteins was furthermore minimized by repeated copious irrigation of the MSC pellet with PBS before final re-suspension and transfer to the operating room.

Cancellous bone grafting (Group II): The operations were performed by the same oral and maxillofacial surgeon. Under general anesthesia, routine draping and sterilization protocol were applied to expose the anterior iliac crest. Incision is made over the anterior iliac crest and deepened to the deep fascia. The musculofascial attachment of the abdominal wall muscles to the iliac crest is defined and any fatty covering should be cleared off to expose the attachment of these fascia. Incision, elevation and reflection of the periosteum was done to expose the superior surface of the iliac bone. Cancellous bone was harvested and collected in a sterile gauze. Wound was closed in layers.

B. Recipient site preparation (Alveolar cleft site) in all patients:

Under general anesthesia, a labial incision was made along the gingival border from the first permanent molar to the contralateral central incisor with vertical releasing incisions. Circular incision around the edges of the cleft was performed separating nasal and oral layers. Nasal mucosa was reflected cephalically to reconstruct nasal floor. Oronasal fistula was closed. Prior to graft insertion bur holes were drilled using surgical round burs in the bone bordering the recipient site to accelerate healing and graft incorporation.

Graft preparation and insertion: For group I, PRP was prepared. 12 ml blood was aspirated and collected into citrated tubes then centrifuged at 3500 rpm for 15 min at room temperature using a multi-speed 4000 rpm vertical rotor. Expanded stem cells were seeded into several cones of collagen sponge (Resorba Company). Nanohydroxyapatite (sigma Aldrich) and PRP were added. The hybrid scaffold was gently packed into the defect figure (1, A). For group II, the cancellous bone chips were packed and condensed into the prepared alveolar cleft site against the reconstructed nasal floor until slight overfilling was achieved. figure (1, B). The gingival mucoperiosteal flaps were sutured. The wound was checked daily for primary wound healing. Antibiotic coverage was administered (Augmentin 100 mg / kg/ day in two divided dose for five days). Ibuprofen syrup was prescribed four times daily for three days. Alpha-Amylase syrup 3 was taken three times daily for three days. Also, nasal decongestant drops were prescribed twice daily for three days in addition to daily repeated use of warm saline mouth rinse. The patient was discharged after 6 days.

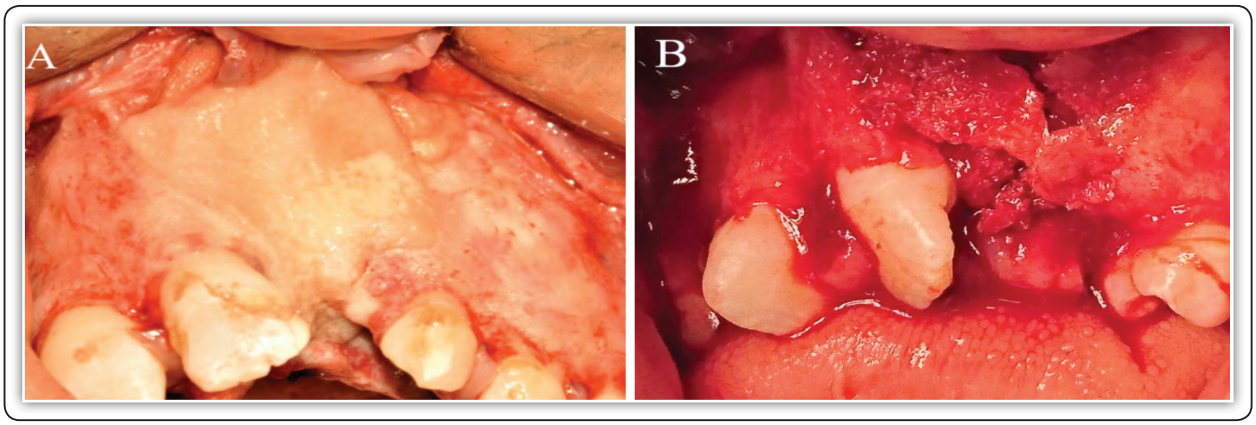

FIG (1) Surgical placement of grafting materials. A- Hybridized scaffold (stem cells seeded in collagen sponge, PRP and nanohydroxyapatite) packed into the cleft defect (Group I). B- Cancellous bone graft placement into the cleft defect (Group II)

III) Follow up evaluation:

Patients were seen on the first postoperative day, every other day for the first week, weekly thereafter for a month then every month for six months. Early postoperative assessment (within one month) was focused on Soft tissue healing regarding the presence of inflammation, purulent discharge and dehiscence from the recipient site. Moreover, postoperative pain by graded visual analog scale [18] (GVAS), gait disturbance, walking problems, changes in the sensibility of the hip were also assessed. Late postoperative assessment (after one month) was focused on persistence of oronasal fistula and tooth eruption.

Statistical analysis: The collected data was revised, coded, tabulated and introduced to a personal computer using Statistical package for Social Science (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22. Armonk, NY: IBM Corp). Data was collected and suitable analysis was done according to the type of data obtained for each parameter. P value ≤ 0.05 is significant (S) while $P < 0.01$ is highly significant (HS) and $P > 0.05$ is non-significant.

RESULTS

Early post-operative, At the recipient site, few patients complained from pain and tenderness in the first post-operative week scored on VAS to be 4 in group I and 5.5 in group II which was relieved by continuing the already prescribed analgesic. The pain disappears gradually within two weeks. At the donor site, no paresthesia, infection or gait disturbances were found in any of the cases. all patients in group I experienced no pain or discomfort at the bone marrow aspiration site while patients in group II experienced pain scored 3 by VAS and disappeared gradually within a week.

Sutures were removed in the 10th day post-operatively. Soft tissue healing was complete except for case number 2 in group II which exhibited a small area of dehiscence and extrusion of bony spicules. This was treated by local wound care using mouth wash for ten days. Systemic antibiotic was also prescribed for five days. Improvement of oral hygiene was stressed on for the child and his parent in addition to adequate nutrition. There was improvement at recipient site after one week of treatment. Analysis of the clinical outcomes for each case is presented in table (1). wound dehiscence represented 14% (as one case) of group II.

Late post-operative, the percentage of preoperative ONF was 75% versus 85% in group II. soft tissue healing was complete at one month postoperatively with successful closure of preexisting oronasal fistula (ONF) in both groups. After six months postoperatively eruption of lateral incisor was observed in two cases in group I (25 %) and one case (14%) in group II as shown in figures (2,3).

TABLE (1): Analysis of the clinical outcomes for each case.ONF (oronasal fistula), +ve (present), -ve (absent).

Case	Dehiscence		ONF Pre		ONF Post		Tooth eruption	
	Gp I	Gp II	Gp I	Gp II	Gp I	Gp II	Gp I	Gp II
1	-ve	-ve	+ve	+ve	-ve	-ve	+ve	-ve
2	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve
3	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve
4	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve
5	-ve	-ve	+ve	+ve	-ve	-ve	+ve	-ve
6	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve
7	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve
8	-ve		+ve		-ve		-ve	
Total	0	1	6	6	0	0	2	1
Ratio	1/15		12/15		0/12		3/15	

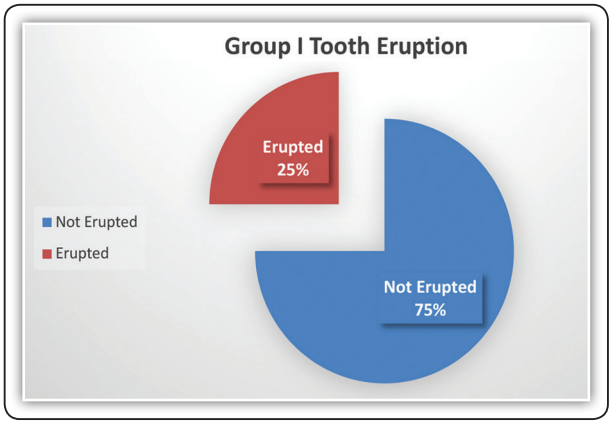

FIG (2-A): Pie chart representing the percentage of tooth eruption in group I

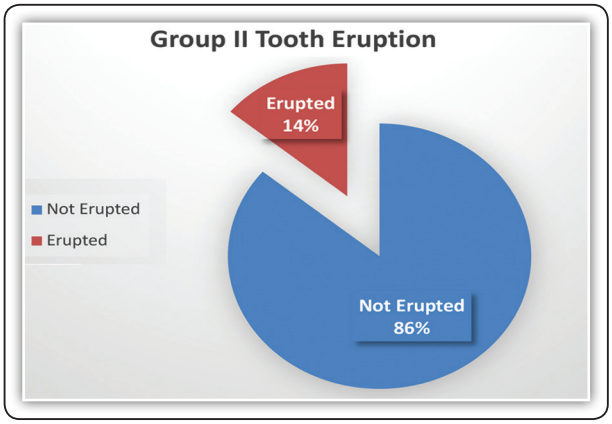

FIG (2-B): Pie chart representing the percentage of tooth eruption in group II

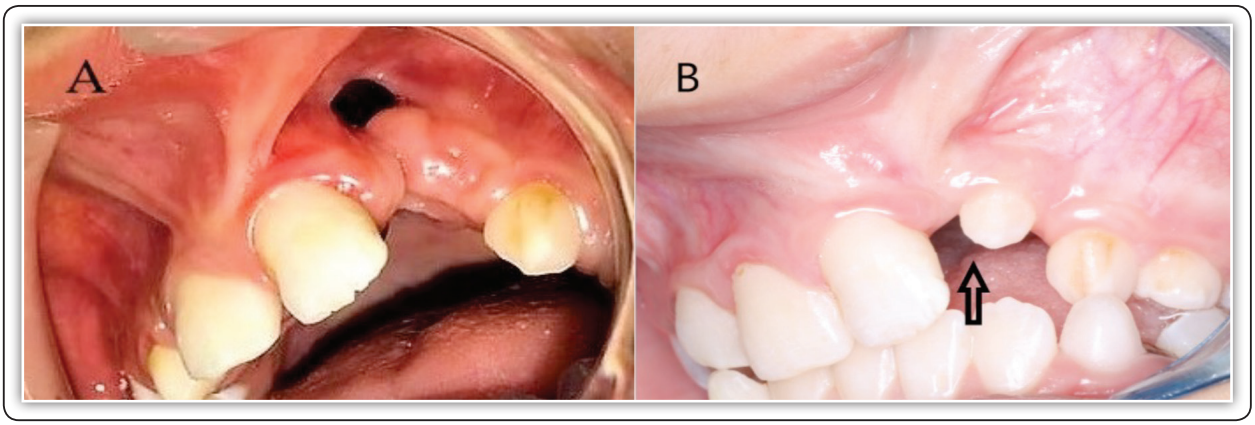

FIG (3): Tissue healing and tooth eruption after 6 months in a case in group I. A- preoperative, B- 6 months postoperative. Arrow indicating erupting left lateral incisor.

DISCUSSION

The ideal treatment of alveolar cleft is not yet settled. Even we achieved good results with cancellous bone grafting which become the gold standard, the technique still has some concerns; it needs another donor site with many possible complications[7]. So, cleft lip and palate surgeons are looking for smart solution and searching for more conservative techniques that fulfill the optimal replacement with less complications.

Alloplastic materials have been introduced to replace the autogenous cancellous bone graft and morbidity of the donor site but with limited success[8-10]. Recently, stem cell tissue engineering is a promising technique for replacing many defective tissues and for alveolar cleft [11,12].

In the present study, we use only unilateral cases to simplify the technique. Bilateral defects are more complicated and may be done in another study. We exclude cases with any other previous attempt to surgical correction to avoid poor soft tissue quality and scaring which may negatively affect the results. we use secondary ACG for the following reasons: first ACG is of great importance prior to the eruption of permanent teeth to provide their stabilization by the pre-grafted bone. Second, ACG has no negative effects on maxillary growth [19].

The iliac crest was selected for aspiration of bone marrow mesenchymal stem cells (BMMSCs) according to previous positive results [20,21]. The technique of multiplication of stem cells was used in many publications and considered as a standard for cell culturing and expansion[16,17,22,23]. The collagen type 1 and nano-hydroxyapatite COLI/n-HA was used as a scaffold for seeding the BMMSCs according to reported positive results in enhanced bone formation by increasing the proliferation and differentiation of osteoblasts in vivo and in vitro [24,25]. The platelet rich plasma (PRP) was used to deliver growth factors to the graft [26]. In the present study, the hybridized scaffold was used to promote biocompatibility and osteogenic induction of BMMSCs.

Different clinical methods have been employed for the evaluation of secondary ACG outcomes, such as postoperative pain assessment, integrity of soft tissue covering the grafted bone and eruption of the teeth adjacent to the cleft site. In the current study, the patients found that postoperative pain at the hip donor site was as or less than they had expected, and the median value for worst pain was 3 on a GVSA of 1–10. Which disappeared gradually within a week. This may be due to delicate manipulation of tissues, careful closure with anatomical respect and small amount of required bone. The findings here concur with previous study of hip donor site morbidity presented by Rawashdeh [18].

Wound dehiscence can lead to direct exposure of the graft to the contaminated oral and/or nasal environments. As a result, infection and resorption of the bone graft with a diminished final bone volume may take place[27]. Wound dehiscence may be caused by over packing the defect with an excessive amount of bone graft, tension in the soft tissue after closure of the wound, local trauma postoperatively, or poor patient compliance with the postoperative oral hygiene [27].

In our study, the incidence of wound dehiscence was only (14%) in group II. This was claimed to over packing of bone graft and poor oral hygiene. In Kubuta et al. [28] study, the incidence of dehiscence in the bone grafting with stem cell group was 20.6% in 18 clefts. It is important to mention that, the patient who had wound dehiscence, was presented with poor oral hygiene. The poor oral hygiene is one of the most important causes of infection that leads to a greater incidence of absorption of the graft as claimed by Borba et al [29].

Soft tissue healing of all cases was complete with successful closure of oronasal fistula because we have not complicated cases, followed the same surgical technique including nasal closure and performed presurgical extraction of supernumerary teeth that may interfere with closure.

A further important goal for bone grafting in alveolar clefts is the eruption of the teeth adjacent to the cleft site. Although, it is a successful long-term outcome of graft survival, it has at the same time a positive effect on bone regeneration and provides support and protection for the adjacent teeth. Pradel et al. [24] have demonstrated the successful use of differentiated osteogenic cells on collagen sponge for cleft repair in 10 years old boy. They observed movement of erupting canine into the newly formed bone Eight months after grafting and spontaneous eruption in its proper place Eighteen months postoperatively. The present study had two female cases in group I aged 8 and 8.5 years and a boy aged 9 in group II with successful eruption of left lateral incisor in cleft area after 6 months of grafting. This result suggest that tissue engineered bone can function like natural bone, allowing for physiologic processes such as spontaneous tooth eruption to occur. Because the tooth eruption needs longer term follow up, Other cases in both groups did not show spontaneous eruption. Although, partial and complete movement of erupting canine in grafted area were observed in some cases.

CONCLUSION

The effect of stem cell tissue engineering did prove to have a positive clinical result compared to gold standard used in alveolar cleft grafting and provided significant support to the healing of soft tissues around the alveolar cleft. Also, Tissue engineering bone graft is a cost effective and require two setting comparable to the autogenous bone graft. However, radiographic assessment was needed to evaluate the bony closure of the cleft.

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