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Cleft palate reconstruction by platelet-rich-plasma and stem cell injection: Histological evidences

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Cleft palate (CP) is a common congenital defect. It causes serious problem to cleft-affected children. The gold standard of care is autogenous bone grafting which may cause additional problems together with long and extensive medical interventions. Tissue engineering is a promising solution for a widespread range of defects and disorders. It is reasonable to utilize this novel technology for CP management. Stem cells and growth factors play essential role in tissue engineering, so we evaluate effectiveness of adipose tissue and cord blood stem cells along with platelet rich plasma (PRP) on CP reconstruction. Human umbilical cord blood mononuclear cells (hUCB-MNCs) and human adipose-derived stem cells (hADSCs) were collected and incubated with Bromodeoxyuridine (BrdU) for labeling. The same was done to osteogenically differentiated hADSCs. Palatal bone defects were surgically made in rats. Afterwards, the labeled cells were mixed either with PRP or Aminoplasmal and injected to the defect borders. Immunohistochemistry and morphometry analysis were performed 4 weeks later. Data showed a significant difference in cleft size between cell-injected and control groups while the cleft site was filled with connective tissue rather than osseous tissue. Moreover, immunohistochemistry findings proved the presence of labeled cells in the surrounding tissue. These cells were detectable both in osseous and connective tissues. This study revealed the feasibility of stem cell and PRP application according to CP reconstruction. Hence, further investigation toward tissue engineering in CP may eliminate bone harvesting and its negative consequences.

Key words: Cleft palate, platelet-rich-plasma, cell therapy, stem cells, tissue engineering.

INTRODUCTION

During weeks 8 to 12 of human intrauterine life, palate is formed (palatogenesis). This structure separates oral and nasal cavities from each other. Palatogenesis is a highly coordinated process, depending on anatomical specific
and precisely timed molecular signals for normal development (Chai and Maxson, 2006). Palatal shelves are two protrusions from lateral walls of the primary oral cavity which fuse in the midline to form the majority of palate named secondary palate. Secondary palate is continuous with the primary palate anteriorly and the soft palate posteriorly (Bush and Jiang, 2012). The interruption in fusion of these segments may cause cleft palate (CP) which is a frequent congenital defect in human kind (Farina et al., 2002). Birth prevalence ranges from 1/500 to 1/2,000 in different races (Marazita et al., 2004).

Cleft palate reconstruction procedure should tackle normal speaking and feeding abilities. At the same time, normal facial appearance should be considered. In this way, one of the major difficulties is bony reconstruction. Therefore, autogenous bone grafts is the gold standard of care of CP now. The most common bone donor sites are tibia, mandible, ilium, cranium and ribs (Batra et al., 2004). Bone harvesting procedure yields limited amount of bone which may not sufficient for grafting and cause failure (Panetta et al., 2008). In addition, bone harvesting procedure is followed by several complications mostly related to the donor site; morbidity is the major problem (Batra et al., 2004). The reported success rate was 41 to 73% after primary grafting; unfortunately most of patients need additional operations (Nguyen et al., 2009). On the other hand, grafting surgery may cause some unwanted consequences such as facial growth disturbance result (De La Pedraja et al., 2000) and mid-face retrusion (Waite and Waite, 1996). Postoperative oronasal fistula development in grafted area still remains a significant challenge of the palatal reconstruction surgery. There is an incidence rate ranging from 11 to 23% (Amaratunga, 1988; Cohen et al., 1991; Emory et al., 1997; Landheer et al., 2010). Allogeneic and synthetic materials are another available choice, but they have some disadvantages as well including risk of infection, immunologic issues, structural integrity and contouring abnormalities (Bostrom and Mikos, 1997). Hence, researches are going on to improve the way cleft palate will be managed in the future. Tissue engineering opens a new sight of view towards many types of defects and disorders management. Cells, growth factors and scaffolds are essential parts of tissue engineering (Logeart-Avramoglou et al., 2005). An optimal cell source is characterized by availability in large quantities, no immune rejection, no graft versus host disease, no tumorigenicity, predictable differentiation potential for stem cells, and integration into the tissues (Logeart-Avramoglou et al., 2005). Mesenchymal stem cells (MSCs) are appropriate choice for tissue engineering (Diao et al., 2009). Different sources of MSCs are available all over the body including umbilical cord blood (UCB-MSCs), Wharton’s jelly (UC-MSCs), bone marrow (BM-MSCs) (Diao et al., 2009) and adipose tissue (ADSCs) (Conejero et al., 2006; Tobita et al., 2008).

The use of MSCs in cleft reconstruction is still rare in clinic. However, autologous and allogeneic BM-MSCs along with hydroxyapatite/tricalcium phosphate scaffold were applied in alveolar cleft models. There was no immunological response to neither autologous nor allogeneic BM-MSCs and it showed both cells had the capacity to regenerate bone within craniofacial defects (Kok et al., 2003). Another study combined autologous BM-MSCs with sponge collagen protein and investigated restoration of the alveolar cleft in dog model by these materials. Results showed this method can be used clinically to treat alveolar cleft (Ou et al., 2007). Likewise, desirable results came from another study which used BM-MSCs seeded into different kinds of scaffolds for alveolar bone repairment in dog model (Mylonas et al., 2007).

Adipose tissue and umbilical cord blood have become extremely attractive options for cell-based therapy to avoid pain and stigma associated with the bone marrow harvesting procedure, and also to achieve greater number of stem cells (Mizuno et al., 2012; Seghatoleslami et al., 2012). Rat fat-derived stem cells seeded onto poly-L-lactic acid scaffold also used to repair rat palatal bone defect models, the feasibility of using these cells for healing such defects was demonstrated (Conejero et al., 2006). Moreover, engineered bone from canine ADSCs and autogenous bone graft were compared on a survey; autogenous bone graft was statistically more effective than engineered bone (Pourebrahimi et al., 2013).

To improve stem cells’ ability to form osteoblasts many studies treat the cells with the osteogenic growth factor BMP2 (Chin et al., 2005; Kang et al., 2011). But they fail to determine levels of osteoinductive effects of BMP2 (Zuk, 2008). In addition, adverse events are reported along with BMP2 application (Woo, 2012). As such, further studies need to find the best growth factors source for regenerative medicine. Platelet-rich-plasma (PRP) is a readily available source of growth factors. Platelet-rich-plasma is blood derivative defined as high concentration of platelets in a small volume of plasma (Marx, 2001). Platelet’s alpha granules contain molecules as platelet-derived growth factor, transforming growth factor β, epidermal growth factor, fibroblast growth factor, insulin-like growth factor I, vascular endothelial growth factor and others. These factors stimulate local inflammation, granulation tissue formation, cell proliferation, cell differentiation, neovascularization and extracellular matrix production which are essential for tissue regeneration (Jamilian et al., 2007; Méndez et al., 2006; Ravari et al., 2011). The rational beyond using PRP in tissue engineering is due to release bioactive factors, which promote angiogenesis and also the extracellular matrix synthesized (Ravari et al., 2011; Shirvan et al., 2013a; Shirvan et al., 2013b). There is a gap in researches on CP reconstruction using stem cells in combination with PRP. However, MSCs have been combined with platelet-rich plasma to heal an alveolar cleft in a 9-yr-old girl, recently (hibi et al., 2006).

The objective of this study is to evaluate usefulness of
hADSCs and hUCB-MNCs along with PRP in cleft palate reconstruction, as an alternative method for bone harvesting.

MATERIALS AND METHODS

Cell isolation and culture

Human adipose-derived stem cells (hADSCs) and human umbilical cord blood mononuclear cells (hUCB-MNCs) are used in this study. All procedures were approved by Mashhad University of Medical Sciences ethics committee, informed consent was obtained from all donors.

The human adipose tissues were obtained by liposuction from the abdominal subcutaneous fat of healthy and non-addicted 30-40 years old women. In order to hADSCs isolation, the adipose tissue samples were washed with phosphate-buffered saline (PBS), and digested by 0.01% type-I collagenase in PBS (1.5 h at 37°C). Thereafter, hADSCs were sedimented at 600 g for 15 min at 25°C, and cultured in αMEM (Gibco, Invitrogen Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Sigma, St. Louis, MO) (Taha and Hedayati, 2010). Non-adherent cells were removed 24 h later; afterward, one culture medium was changed every third day. Cells were passaged at 70% confluency using trypsin-EDTA (Gibco, Invitrogen Carlsbad, CA). The cells from third passage were harvested, their viability estimated by means of a Neubauer hemocytometer plate and Trypan blue dye exclusion method. These cells were used for characterization by flow cytometer in vivo experiments and osteogenic differentiation (Rada et al., 2012). For in vivo experiments, hADSCs and osteoblastic differentiated cells were labeled with 3 µg/ml bromodeoxyuridine (BrdU) for 48 h.

Umbilical cord blood was collected from umbilical cord after childbirth. Mothers were healthy, non-addicted, and ranging in age from 20 to 40. Standard blood collection bags containing citrate phosphate dextrose adenine (CPDA) were used for this purpose. Cell isolation was done by density gradient technique using Ficoll-Paque solution (Sigma-Aldrich, Inc., St. Louis, MOUSA), and centrifuged at 800 g for 20 min at 25°C. Mononuclear cells (MNCs) layer, theuffy coat, was carefully collected and washed twice with PBS. Then, the cells were re-suspended in fetal bovine serum (10%) supplemented RPMI medium (both from Gibco, Invitrogen Carlsbad, CA) plus 3 µg/ml BrdU, which labels DNA, and incubated in CO2 incubator at 37°C. After 24 h the cells were harvested and re-suspended in UCB serum for in vivo experiment (Seghatoleslam et al., 2012). The hUCB-MNCs viability was assessed and flow cytometric characterization was done as well.

Cell characterization

With the aim of hUCB-MNCs characterization, the expression of hematopoietic stem cells surface markers (CD45 and CD34) were evaluated (Seghatoleslam et al., 2012). Mouse anti-human-CD45-FITC and mouse anti-human-CD34-PE specific monoclonal antibodies (AbD Serotec, Inc., Endoveaur House, Kidlington, Oxford, UK) were used.

As stated by others, hADSCs was positive to several CD markers including: CD9, CD29, CD49, CD54, CD105, CD166, CD44, CD71, CD10, CD13, CD73, CD90, CD59, CD146 and CD55 (Taha and Hedayati, 2010). Here we assessed CD29 and CD90 cell surface markers expression rate by mouse anti-human-CD29-FITC and mouse anti-human CD90-PE (AbD Serotec, Inc., Endoveaur House, Kidlington, Oxford, UK).

Briefly, samples were incubated with antibody for 20 min at 25°C in the dark, washed with PBS and run through a BD FACS machine (BD Biosciences, San Jose, CA) which was tuned with respect signal amplification for fluorescein isothiocyanate (FITC) and phycoerythrin (PE) (FL2). The isotype control antibodies were used for confirmation. Data analysis was performed by WinMDI 2.9 software.

Osteogenic differentiation

At the third passage of hADSCs osteogenic differentiation induced by a media contained 100 nM dexamethasone, 5 µM ascorbic acid, 0.5 mM β-glycerophosphate, and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Differentiation occurs after day 14, and a week after that almost all cells will show osteoblast properties (Desai et al., 2013).

Confirmation of osteogenic differentiation

Osteogenic media was removed after 21 days, and cells were washed with PBS and fixed with paraformaldehyde 4%. Afterward, the fixative was eliminated, alizarin red solution was added, and the cells were incubated at 25°C for 20 min, washed with excess distilled water. Finally, the cells were scanned by a microscope (Olympus, BX51, Japan) attached to a digital camera (Canon, IXUS 950 IS).

Preparation of activated hPRP

A 450 ml blood sample was obtained from blood bank of Mashhad, Iran. The two steps centrifugation method was carry out to remove erythrocyte fraction, and then platelets were sedimented. Subsequently, platelets were re-suspended in appropriate volume of plasma. In order for platelet activation, several freezing and thawing cycles were done; then hPRP was stored at -70°C until use (Mishra et al., 2009).

Surgical procedure

Sixty four 2-months-old female Wistar rats, weighting about 200-250 g, were housed at a constant temperature of 24.5°C and fed normally. All institutional and national guidelines for the care and use of laboratory animals were followed. The rats were anesthetized with Ketamin (80 mg/kg) and Xylasine (5 mg/kg) intraperitoneally. A critical size bony defect was made in the midline of palate, posterior to the upper incisors and anterior to palatal rugae, using a surgical trephine driven by a low-speed dental engine (Takano-Yamamoto et al., 1993). Animals were divided into eight groups (n=8) on the basis of the injected materials to the defect borders: 1) Group N, normal saline; 2) Group P, PRP without cells; 3) Group G, aminoplasmal without cells; 4) Group AP, undifferentiated hADSCs with PRP; 5) Group OP, osteogenically differentiated hADSCs with PRP; 6) Group AA, undifferentiated hADSCs with aminoplasmal; 7) Group OA, osteogenically differentiated hADSCs with aminoplasmal; 8) Group UP, UCB-MNCs with PRP. Each rat received just one injection immediately after surgery. The volume of each injection was 300 µl contained 4×10⁶ cells. The recovery after operations was uneventful and animals continued to feed normally.

Computerized morphometric measurement

Animals were sacrificed 4 weeks after injection and palates harvested. Images were taken from samples using a digital camera (Canon, SX260 HS), a ruler was used as scale bar in the images.
Immunohistochemistry

Palates were fixed with 10% formalin for 4 days, decalcified with 40% acid formic in PBS for 3–4 weeks, dehydrated, and embedded in paraffin. The paraffin blocks were cut in to 7 μm thickness. The sections were deparaffinized, rehydrated and used for immunohistochemistry staining. Heat mediated antigen retrieval was used to unmask antigens. After that, permeabilization was carried out with 1% BSA and 1% triton X, endogenous peroxidase activity was blocked with 3% H2O2 in methanol for 15 min at room temperature, then non-specific antigen blocking was preformed with PBS containing 1% goat serum. Afterward, sections were incubated with Anti-BrdU or Anti-human osteopontine as primary antibodies and anti-mouse IgG peroxidase conjugated as secondary antibody. Finally, dianamobenzidin (DAB) chromagen was applied to visualize antigens. Counterstaining was done with hematoxylin, cover slipped and scanned by BX51 microscope (100X) attached to a digital camera (Canon, IXUS 950 IS). Cells labeled with BrdU were counted using freely available Image J 1.47v software.

Statistical analysis

Statistical analysis was performed by SPSS 11.5 software (SPSS, Inc., Chicago, Ill.) and one way ANOVA used to compare groups followed by Post Hoc Tukey test.

RESULTS

Flow cytometry analysis

The results obtained from flow cytometry analysis of the hADSCs after the third passage is shown in Figure 1 which confirmed the expression of both CD29 and CD90. The mean percentage of expression was 95.25 and 99.61%, for CD29 and CD90, respectively. As can be seen in Figure 2, the flow cytometry analysis of the hUCB-MNCs indicates that CD45 and CD34 cell
Osteogenic differentiation

While osteogenic differentiation takes place cells start to deposit calcium ions in the extra cellular matrix. These nodular calcium depositions are detectable by specific stains like Alizarin red. This is illustrated in Figure 3 that surface markers were expressed among gaited cells. The mean percentage of expression was 89.43% for CD45 and 20.99% for CD34.

calcium depositions were stained red while cells remain unstained by Alizarin red staining.

Morphometric analysis

Results show the effectiveness of SCs and PRP injection for palate defects reconstruction (Figure 4). This results is considered as significant at the P<0.05 level. Image analysis demonstrates withdrawal of defect edges and bone resorption in control group (N) which had wide
defects. Conversely, all other groups had significant smaller defects in comparison with N. From the data in Figure 4, it is apparent that cell administration regardless the source and differentiation level had significant effect on cleft closure (P<0.001). We did not find any significant difference between PRP and aminoplasmal as carrier, but both were significantly different as compared to N (P<0.01 and P<0.05 for P and A, respectively). It is also shown in Figures 4 and 5 that all experimental groups had statistically significant effect to reduce defect size in comparison with P and A groups (P values are mentioned on Figure 5) (all data in each group passed normality test before ANOVA analysis). Our study did not show any significant difference between differentiated and undifferentiated cells (Figure 5).

Immunohistochemistry

In cell treated groups, BrdU+ cells were detectable at cleft site 4 weeks after injection (Figure 6). Microscopic scanning of stained sections revealed that the defects were filled with connective tissue rather than bony tissue, but the labeled cells were also seen in surrounding bone. The cell counts are summarized on Figure 7. There were significant differences between hADSCs treated groups and hUCB-MNCs (P<0.01), but there were no differences among other groups.

Immuno-staining for human osteopontin confirmed the expression of this protein and its presence in extra cellular matrix on all cell treated groups (Figure 8).

DISCUSSION

The current study showed that stem cells along with PRP could have a positive effect on cleft palate reconstruction. Surprisingly, stem cell application along with aminoplasmal had almost same effect. Contrary to expectations, this study did not observe statistically significant difference between two stem cell types applied in the survey. Another important finding is that osteogenic differentiation seems to have no effect on palate reconstruction procedure.

As mentioned previously, there are several disadvantages related to surgical treatment of cleft palate. In order to address these challenges, tissue engineering had been proposed as an alternative therapy.
(Conejero et al., 2006; Pourebrahim et al., 2013). However, there are a limited number of experimental studies in this field. Besides, the majority of studies have tried to enhance the standard treatment outcomes by PRP application rather than establish a new method. Therefore, in attempt to use tissue engineering for cleft palate reconstruction, we applied ADSCs and also UCB-MNCs along with PRP.

Platelet-rich-plasma as a storage vehicle of growth factors is applied recently in tissue engineering. The growth factors are platelet-derived growth factor, transforming growth factor-b1 and -b2, platelet-derived epidermal growth factor, platelet-derived angiogenesis factor, insulin growth factor-1 and platelet factor-4 have influence on bone regeneration. In addition, basic fibroblast growth factor, epithelial cell growth factor, interleukin-1 and osteonectin (major protein in mineralization) in the alpha-granules of the platelets (Aghaloo et al., 2002; Marx et al., 1998; Marx, 2004; Tozum et al., 2003). This implies that PRP may influence bone formation through a variety of pathways. Others revealed that PRP stimulated proliferation of bone cells derived from human trabecular bone (Gruber et al., 2002) and rat bone marrow (Oprea et al., 2003). Some studies revealed positive effects of PRP on bone regeneration in combination with bone grafts (Ohva et al., 2005). Additional studies revealed that PRP had a stimulating effect on the initial cell growth and matrix mineralization of rat bone marrow cells (Dolder et al., 2006).

Many studies have been performed on combinations of PRP and mesenchymal stem cells, in vitro (Ccho et al., 2011; Dohan et al., 2010; Mishra et al., 2009; Xie et al., 2012). Almost all of these studies' results showed that PRP increased cell proliferation but divergences were found regarding the stem cell differentiation capacity to osteoblasts (Dohan et al., 2010) or chondroblasts.
(Drengk et al., 2009; Mishra et al., 2009). This could be due to the differences between PRP preparation methods. In addition, some other surveys showed supportive results toward usage of PRP in wound healing and tissue regeneration due to high concentrations of PDGF and TGF are secreted from platelet α-granules after activation. These growth factors are described as mitogenic and attractant for mesenchymal stem cells, which may mediate regenerative effects (Amable et al., 2013; Eto et al., 2011; Roubelakis et al., 2014).

Judging by the PRP composition and its effects described here, our findings support the use of PRP along with stem cells in CP regeneration. Our study revealed the promising effects of PRP in cleft palate closure. This may be due to elimination of bone resorption and also to aid tissue regeneration. Also, this finding is in agreement with Hibi et al. (2006) which used a mixture of MSCs and PRP for an alveolar cleft osteoplasty in a 9-year-old female patient successfully. Nevertheless, results remain highly controversial due to lack of studies that assessed PRP and stem cell effectiveness on reconstruction of maxillary defects, simultaneously. Despite this, there is limited number of surveys towards clinical application of PRP in maxillary auto-graft surgery which leads to arguable subject (Luaces-Rey et al., 2010; Marukawa et al., 2011).

The rationale behind using stem cells is their ability to differentiate into a wide range of cell types. Considering of tissue engineering principles of stem cells could have the essential role in angiogenesis and osteogenesis. It is obvious that well-established tissue nutrition improves the regeneration (Kanczler and Orefo, 2008). Adipose tissue is a readily available source of stem cells in body. Adipose derived stem cells have been demonstrated to differentiate into osteoblasts, chondrocytes, adipocytes, myocytes and neurons, depending on the microenvironment, in vitro (Gimble et al., 2007). In addition, umbilical cord blood is an abundant source of pluripotent stem cells. The isolated cells from this tissue have been shown to have the capacity to differentiate into different cell lineages (Harris and Rogers, 2007). Furthermore, PRP is utilized in tissue engineering due to its bioactive factors stored in platelet granules. These bioactive factors play important roles in cell proliferation, cell differentiation and angiogenesis (Shirvan et al., 2013a).

This study produced results which corroborate the findings of previous studies in this field (Conejero et al., 2006; De Kok et al., 2003). Conejero et al. (2006) made a surgical defect on rat palate and tried to repair it by osteogenically differentiated ADSCs seeded onto poly-L-lactic acid scaffolds. The samples were harvested 6 or 12 weeks after treatment. Results demonstrated the feasibility of reconstructing bony defects with differentiated ADSCs. Although there was newly formed bone in differentiated cell treated group, but they found connective tissue in undifferentiated cell treated animals (Conejero et al., 2006). Likewise, our data showed connective tissue in all cell treated animals.

**Figure 7.** Cell count among groups (Group UP, PRP with hUCB-MNCs; Group AP, PRP with undifferentiated hADSCs; Group AA, aminoplasma with undifferentiated hADSCs; Group OA, aminoplasma with osteogenically differentiated hADSCs; and Group OP, PRP with osteogenically differentiated hADSCs)**:**

**Figure 8.** Immunohistochemistry staining against osteopontin. Arrows show stained extra cellular matrix (Group UP, PRP with hUCB-MNCs; Group AP, PRP with undifferentiated hADSCs; Group AA, aminoplasma with undifferentiated hADSCs; Group OA, aminoplasma with osteogenically differentiated hADSCs; and Group OP, PRP with osteogenically differentiated hADSCs).
Unfortunately, our assessments was done only at 4 weeks following the injections; as we demonstrated the presence of cells and osteopontin in the area it could be possible to find bony material after 6 or more weeks.

Further supporting data comes from other studies which applied other types of stem cells to regenerate alveolar defects including BMSCs, PDLSGs (Kim et al., 2009), autologous and allogeneic BMMSCs (De Kok et al., 2003; Ou et al., 2007).

This experiment did not detect any significant difference between differentiated and undifferentiated cells. This is in contrary with previously mentioned study which determined differentiated cells more effective (Conejero et al., 2006).

While this study presents promising data towards cell therapy for cleft palate closure, it failed in addressing bony tissue defect. However, an earlier study compared tissue regeneration of engineered bone with ADSCs and autogenous bone graft in bilateral maxillary alveolar cleft model. Their results found bone autograft significantly more effective than engineered bone (Pourerebrahim et al., 2013).

What is surprising is that aminoplasmal as an injectable mixture of amino acids showed a therapeutic effect. Unfortunately, there was no comparable study in this field. It is remarkable that we choose aminoplasmal as a carrier to omit PRP's association effects, but surprisingly we found it effective itself. So, one of the limitations of this study is the absence of groups with stem cells treatment without PRP or aminoplasmal.

Conclusion
This study proved that the stem cells along with PRP could enhance cleft palate closure in rat models. It seems that the presented method could apply for those cases with small defects to eliminate bone grafting consequences; also PRP and stem cells as adjuvant with autografting may be effective in wide defects. However, caution must be applied towards clinical administration unless more researches are done on this subject. Further work needs to be done to set the exact procedure for clinical use, and also to determine the stem cells effects without PRP or aminoplasmal.

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Conflicts of interest
Authors declare that they have no conflict of interest.

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