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Improved bone regeneration through amniotic membrane loaded with buccal fat pad-derived MSCs as an adjuvant in maxillomandibular reconstruction

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ABSTRACT

Background: Human amniotic membranes (HAMs), as a biological membrane with healing, osteogenic, and cell therapy potential, has been in the spotlight to enhance the outcomes of treating bone defects. Present study aims to clinically assess the potential of HAM loaded with buccal fat pad-derived stem cells (BFSCs) as an osteogenic coverage for onlay bone grafts to maxillomandibular bone defects.
Materials and methods: Nine patients with jaw bone defects were enrolled in the present study. The patients were allocated to two study groups: Iliac crest bone graft with HAM coverage ($n = 5$), and Iliac bone grafts covered with HAM loaded with BFSCs ($n = 4$). Five months following the grafting and prior to implant placement, cone beam computed tomography was performed for radiomorphometric analysis.
Results: The mean increase in bone width was found to be significantly greater in the HAM + BFSCs group (4.42 ± 1.03 mm versus 3.07 ± 0.73 mm, $p < 0.05$). Further, the changes in vertical dimension were greater in the HAM + BFSCs group (4.66 ± 1.06 mm versus 4.14 ± 1.03 mm, $p > 0.05$).
Conclusion: Combined use of HAM with mesenchymal stem cells may enhance bone regeneration specifically in the horizontal dimension. Moreover, this methodology reduces the amount of harvested autogenous bone and diminish secondary bone resorption.

1. Introduction

Decades of research in bone tissue engineering (BTE) have led to a range of treatment approaches. However, in spite of significant advancement in the field, there are many challenges still need to be addressed to achieve efficient clinical translation of BTE approaches [1–4]. A central challenge in bone regeneration is to engineer optimal constructs as a favorable microenvironment for implanted and native cells. Such constructs should ideally support vascularization and enhance the function of implanted cells to effectively restore lost tissue.

Vascularized and non-vascularized bone grafts are the two major categories utilized for reconstruction of critical-sized maxillofacial bone defects. Harvesting a non-vascularized graft is a more simple procedure comparing to vascularized bone graft harvest (Elsalanty and Genecov, 2009); however, it is estimated to undergo 87–105% surface resorption in six years (Sbordone et al., 2012). Vascularized bone grafts experience less bone resorption and have a remodeling similar to the natural bone, but they require further training and specific equipment (Elsalanty and Genecov, 2009). Overall, autografts have limited available sources and are associated with donor site morbidity. Accordingly, tissue engineering was established to develop personalized tissues specifically designed for the defect, and also to overcome the hurdles associated with conventional autografts (Khojasteh et al., 2012). Bone engineering has been applied in several studies, which demonstrated up to 41.34% bone regeneration in sinus augmentation

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(Shayesteh et al., 2008), and 51.3% bone formation in alveolar cleft (Behnia et al., 2012); however, lack of vascularity and limited bone formation has necessitated further research to enhance the clinical outcomes (Khojasteh et al., 2016, 2017). Since scaffolds are a key role player in tissue engineering triad influencing both cell and growth factor delivery (Jafari et al., 2017; Bastami et al., 2017), several modifications have been implemented into their structure to enhance the outcomes, such as manufacturing smart scaffolds (Motamedian et al., 2015), and incorporating extra-cellular matrix into their structure (Jafari et al., 2017; Bastami et al., 2017).

Human amniotic membrane (HAM) is the inner segment of the fetal membranes which includes three layers of epithelium, basement membrane and collagen stroma (Niknejad et al., 2008). Amniotic membrane has several properties that may make it an appropriate scaffold; its extra-cellular matrix possess several growth factors such as transforming growth factor-β, basic fibroblast growth factor (Shimazaki et al., 1997), epidermal growth factor, transforming growth factor-α, keratinocyte growth factor (Koizumi et al., 2000). This tissue is thin and exchanges nutrients by diffusion (Wilshaw et al., 2006), while cell viability due to lack of nutrient in the center of conventional scaffold is one of the major concerns in cell delivery by these structures (Bastami et al., 2017). HAM is demonstrated to enhance wound healing and modulate angiogenesis (Toda et al., 2007), and is demonstrated to enhance wound repair and hemostasis (Gomes et al., 2001). Accordingly, it has long been used as a biologic dressing (Faulk et al., 1980), in ophthalmology surgeries (Gomes et al., 2005), and recently its potential has been highlighted in bone surgeries (Gindraux et al., 2013). There are several *in vitro* and *in vivo* studies supporting the osteogenic capability of HAM. Simultaneous delivery of autografts and amniotic membrane demonstrated 49.23% bone formation in rat femoral critical-sized defects (Starecki et al., 2014), and also co-application of adipose derived stem cells with HAM in periodontal defects demonstrated promising results (Wu et al., 2016). Recently, we have demonstrated that co-application of scaffolds with buccal fat pad derived stem cells (BFSCs) over autogenous bone grafts further enhance bone regeneration and decrease graft resorption (Khojasteh and Sadeghi, 2016). In addition, BFSCs have demonstrated great bone formation and their simple and non-invasive harvest have put them in the spotlight for regenerative approaches (Salehi-Nik et al., 2017). According to the literature on efficacy of HAM and BFSCs in bone regeneration, in the current research we aimed to assess the efficacy of a potentially osteogenic membrane by loading BFSCs on HAM in augmentation of extensively deficient jaws (Scheme 1).

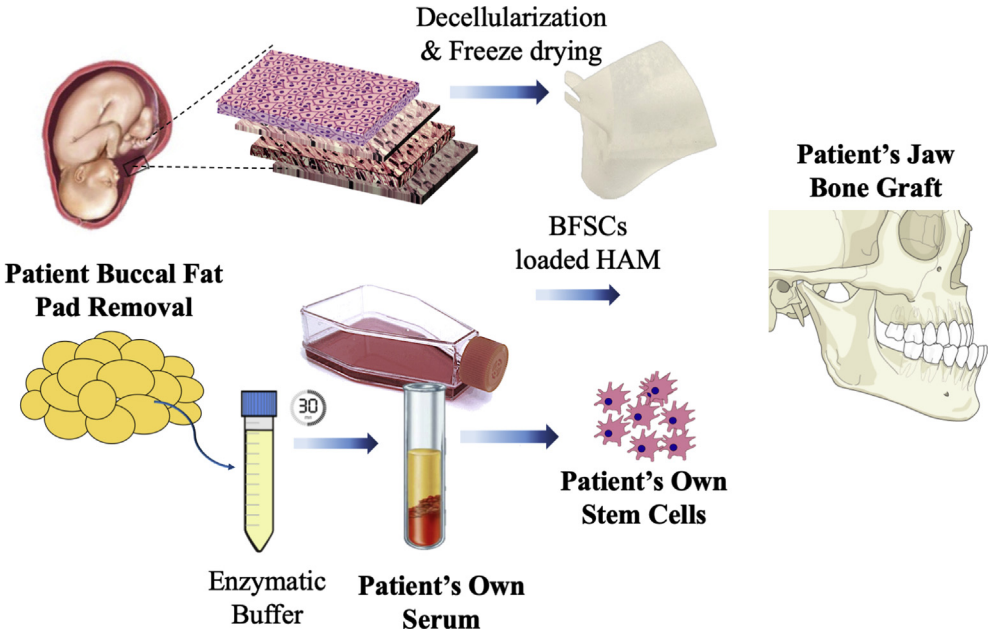
2. Materials and methods

2.1. Subject selection

The present study was carried out at the Department of Oral and Maxillofacial Surgery, Shahid Beheshti University of Medical Sciences, Tehran, Iran. The subjects were selected between the patients were referred to the department. Nine patients with age range between 19 and 53 and normal health condition based on ADA I classification were included in the study. The patients were non-smoker and they were not suffering from any condition conflicting wound healing or would interfere with surgery. Each patient had an extensive bone defects greater than 6 cm in jaws which need a large graft with extra oral donor site bone harvesting. Informed consent was obtained from all the patients according to the approved study protocol by ethics committee at Shahid Beheshti University of Medical Sciences. The patients were allocated into two treatment groups; 4 to the treatment and 5 to the control group. Bone blocks were harvested from anterior iliac crest for restoring the bone defects. In the test group, the anterior iliac crest onlay graft was covered with 2 × 2 cm HAMs loaded with 10^6 BFSCs (BFSCs + HAM group), while in the control group, HAMs lacking any cellular component was used as a protective membrane over the onlay graft (HAM group).

2.2. Isolation and 2D culture of BFSCs

Harvesting buccal fat pad tissue and isolation of BFSCs was performed according to our previous protocol (Khojasteh and Sadeghi, 2016). The autogenous buccal fat pad tissue was harvested from the patients enrolled in the study. The tissue was harvested by a vestibular incision distal to the maxillary second molar. 3–5 ml of tissue was harvested and delivered to the laboratory for cell isolation in chilled filtered PBS. The tissue was rinsed by PBS and minced and incubated in 3 mg/ml type I collagenase PBS (Gibco, NY, USA) for



Scheme 1. The schematic representation of the study design.

30 min at 37 °C. The fragments were then transferred to 25-cm^2 culture flasks containing DMEM (Gibco, NY, USA) with 10% extracted autogenous human serum and incubated at 37 °C with 5% CO_2. The cells were passaged when they reached more than 80% confluency. The 3rd passage cells were used for the experiment.

2.3. Serum isolation from human peripheral blood

In the present study, fetal bovine serum (FBS) was replaced with autogenous human sera. Accordingly, 20 ml of whole blood was obtained from each patient and collected into two 10-ml tubes lacking any coagulant (BD, Plymouth, UK) and rest for clot formation for 30 min at room temperature followed by centrifuge at 2800 g for 15 min at 4 °C. The sera were collected and preserved at −20 °C.

2.4. Assessment of mesenchymal stem cells (MSCs) phenotype of the isolated cells

MSCs phenotype of the isolated cells was verified according to the international society for cellular therapy (ISCT) criteria (Rojewski et al., 2008). To do this, the cells were subjected to flow cytometry to assess expression of CD44, CD90, CD73, CD105, CD45 and CD34 surface markers. The cells at P3 were treated with 0.05% trypsin-EDTA and centrifuged and the cell pellets were re-suspended in PBS at concentration of 105 per sample. The cells were incubated with Fluorescent Isothiocyanate (FITC)-conjugated monoclonal antibodies and phycoerythrin (PE)-conjugated monoclonal antibodies (anti-CD44 FITC, anti-CD90-FITC, anti-CD73-PE, anti-CD105-PE, anti-CD45-FITC, and anti-CD34-PE) (EXBIOPraha, a.s., Vestec, Czech Republic) at 2 μg/ml concentration for 30 min at 4 °C in a dark room. Following the incubation period, the cells were rinsed with PBS and assessment was performed by flow cytometer (BD FACS Calibur; BD Biosciences, Franklin Lakes, NJ, USA). The cells stained with FITC-labeled immunoglobulin G (IgG) were considered as negative control. Analysis of flow cytometry standard files was performed by FlowJo 7.6.1 software (FlowJo LLC, Ashland, OR, USA). Specimen with greater than 90% fluorescent cells was considered positive. Cells at P3 were also assessed for osteogenic capability as follow: cells at density of 104 were cultured in 48-well plates in osteogenic medium consisting of DMEM-LG, 10% FBS, 50 μg/mL ascorbate-2 phosphate, 10−8 M dexamethasone, and 10 mM β-glycerophosphate for 14 days. Alizarin Red staining was then used for determining mineral deposition.

2.5. Human amniotic membranes

The frozen HAMs, pre-tested for mycoplasma and viruses (*e.g.* hepatitis and HIV), have been used in this study. The frozen HAMs were obtained from healthy donors during cesarean section under sterile conditions. The mothers were all tested negative for human immunodeficiency virus type 1 and 2, hepatitis B and C and syphilis. Informed consent was obtained from the mothers for obtaining the chorion. Separation of amnion from chorion was performed by blunt dissection. The tissue was treated with 200 ml of 1% Triton X-100 for 2 h followed by rinsing with 200 ml distilled water twice for 15 min. The tissues were then preserved in 200 ml of 0.1% sodium dodecyl sulfate (SDS) for 10 h on a shaker. The tissues were then lyophilized and stored in freezer.

2.6. 3D culture of BFSCs on HAMs

For *in vitro* study, HAMs tissue was punched to the diameter of the 48-well plates. $l0^6$ BFSCs were seeded over the HAM and the construct was cultured in DMEM+10% human serum. Cell attachment was further verified by observation by light microscope after 24 h and at 3 days by scanning electron microscope (SEM, (Vega, Tescan, PA, USA).

In order to prepare the samples for SEM imaging, the BFSCs loaded membranes were taken out from culture media, rinsed with phosphate buffered saline (PBS) and fixed using fixative solution. Sample were washed with PBS again and dehydrated in gradient ethanol (30, 50, 70, 80, 95 and 100 v/v%). The imaging was conducted after complete drying of the BFSCs loaded membranes and gold coating with Sputtering Coater. The cellular viability and proliferation on the membrane was monitored by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) at 24, 48 and 72 h. For MTT assay, the cell medium was replaced with fresh medium supplemented with 10% MTT solution (5 mg/ml) and incubated for 2 h at 37 °C and 5% CO_2. Afterward, the medium was replaced with dimethyl sulfoxide (DMSO) and read by ELISA reader at 570 nm.

2.7. Surgical procedure

A vestibular incision was made 10 mm away from the crest in the molar-to molar area without any releasing incisions. Buccal and lingual flaps were gently elevated with subperiosteal dissection. A monocortical autogenous bone graft was obtained from the medial surface of the anterior iliac crest and the graft was cut to multiple pieces according to the recipient site's defect and fixed with 1.2-mm microscrews. The natural bovine bone mineral (NBBM, Cerabone, Botiss, Germany) was also used to fill the space between the blocks. In the test group, the onlay graft was covered with HAM loaded with BFSCs while in the control group HAM without any cellular component was used as the graft coverage. According to the ethic approval protocol, Cefazolin (1 g) injections were prescribed prior to the surgery and continued 3-days following the procedure.

2.8. Clinical evaluation and radiographic assessments

Healing sequence of the grafted tissue and soft tissue were evaluated every 2 weeks. Radiographic stents were fabricated for each patient and computed tomography images (CBCT) were obtained before the surgery and before implant insertion. The patients were instructed to wear their stent during imaging and bone width and height were measured in six edentulous areas (A, B, C, D, E and F) and at three levels (1, 3- and 6-mm depth) from the crest of the atrophic ridge (BW1 and BH1). Hence 72 sites were assessed in test group and 90 sites in the control group. Five months following the graft surgery, implants were inserted and the second CBCT was obtained before the implant surgery. While wearing stents during imaging, the bone width and height were measured at the respective sites (BW2 and BH2) and the changes in bone was measured by calculating BW2-BW1 and BH2-BH1.

2.9. Histological analysis

2-mm trephine biopsies were obtained during implant surgery from space between bone blocks that had been filled with NBBM filler. The specimens were fixed by preservation in 10% buffered formalin for 7 days and decalcification was performed by formic acid and sodium citrate for 24 h following rinsing with tap water and dehydration with various concentration of ethanol, the specimen was cleared in xylene (Sigma Aldrich), infiltrated by paraffin and 7 μm sections were stained by hematoxylin and eosin (H&E) (Behnia et al., 2009a; Khojasteh et al., 2019). The specimens were visualized by a light microscope (Olympus SZX9, Olympus, Tokyo, Japan) and assessed regarding presence of inflammation, remnants of HAM and bone formation. Percentage of new bone formation was analyzed by computerized image analysis software (ImagePro Plus; Media Cybernetics, Silver Spring, MD, USA).

2.10. Data analysis

Continuous and discrete variables were expressed as mean and frequencies, respectively. The ANOVA test was used for analysis and data analysis was performed by IBM SPSS version 24.0 (IBM Corp., Armonk, NY, USA). Probabilities of 0.05 or less were considered significant.

3. Results

3.1. 2D BFSCs cellular assessment

Stem cells isolated from the patient's buccal fat tissue and cultured with autologous serum have revealed to express mesenchymal stem cell markers and ability toward osteogenic differentiation. The schematic extraction protocol of stem cells from buccal fat pad tissue was shown in Fig. 1A. Isolated stem cells were investigated for their cellular surface markers to assure the MSCs phenotype. Our obtained results from fluorescence activated cell sorting analysis presented in Fig. 1. The results demonstrated that more than 95% of the cells expressed CD44, CD90, CD73 and CD105 while they were negative for CD45 and CD34 hematopoietic markers. The isolated stem cells have formed fibroblast-like morphology (Fig. 1B). After 14 days osteogenic differentiation, Alizarin Red S staining, as a functional assay, was used on cultured BFSCs to assess their differentiation capacity. As evident in Fig. 1C, osteogenic differentiation of BFSCs is confirmed with the positive Alizarin Red S staining.

3.2. 3D cultured BFSCs attachment and proliferation on HAMs

The HAM is consisted of the main layers of epithelium, basement membrane, compact and fibroblast layer (Fig. 2A) [10, 11]. The decellularization and freeze-drying of the membrane preserve the

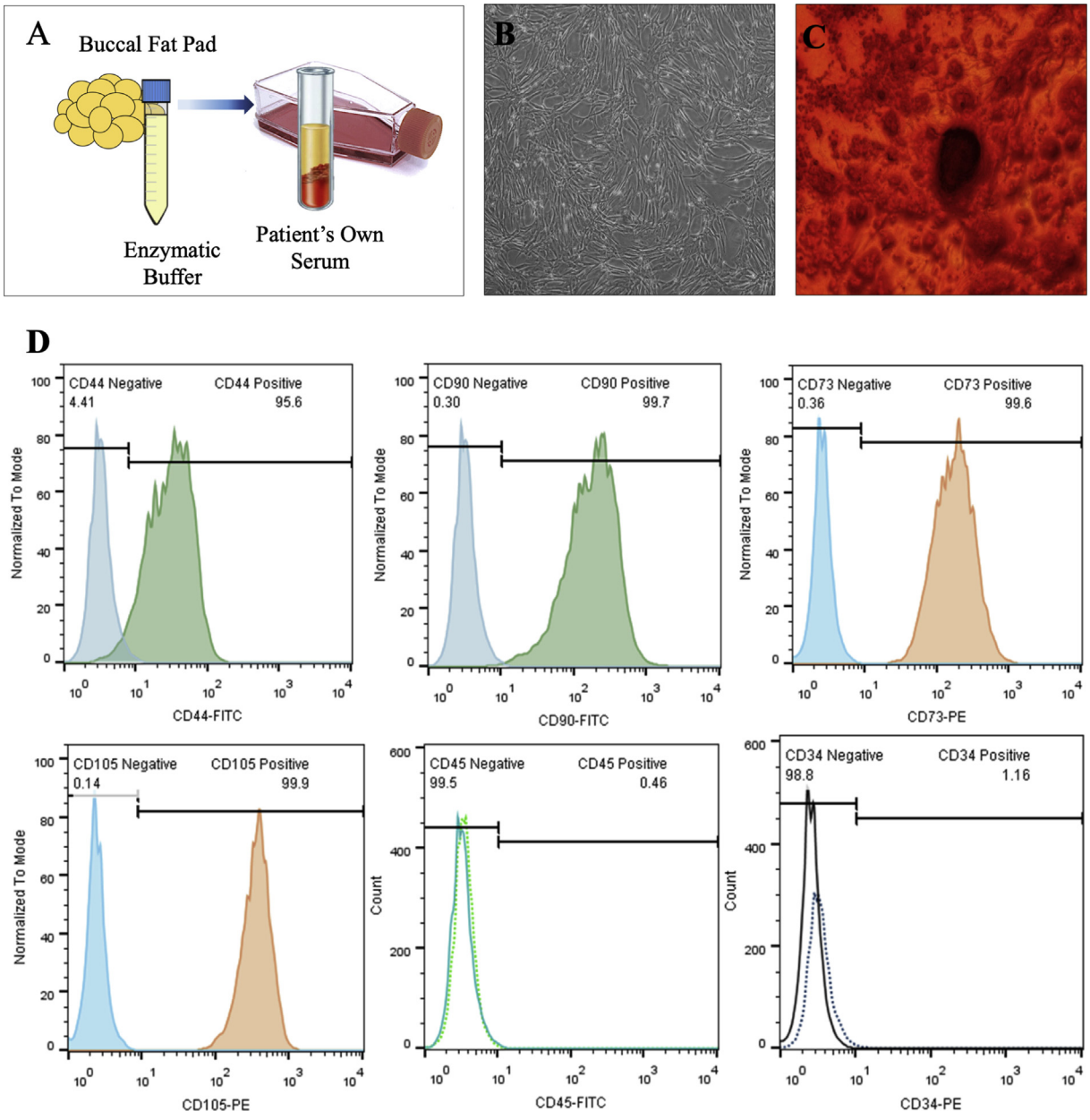


Fig. 1. (A) The schematic representation of the stem cells isolation from fat tissue (B) Isolated stem cells from fat tissue showed fibroblastic morphology (C) Alizarin red staining of BFSCs at P3 in osteogenic medium verified their osteogenic differentiation. (D) Characterization of primary cultures of the BFSCs by flow cytometry. Note that the cells expressed markers for CD44, CD90, CD73 and CD105 while they were negative for CD45 and CD34 hematopoietic markers.

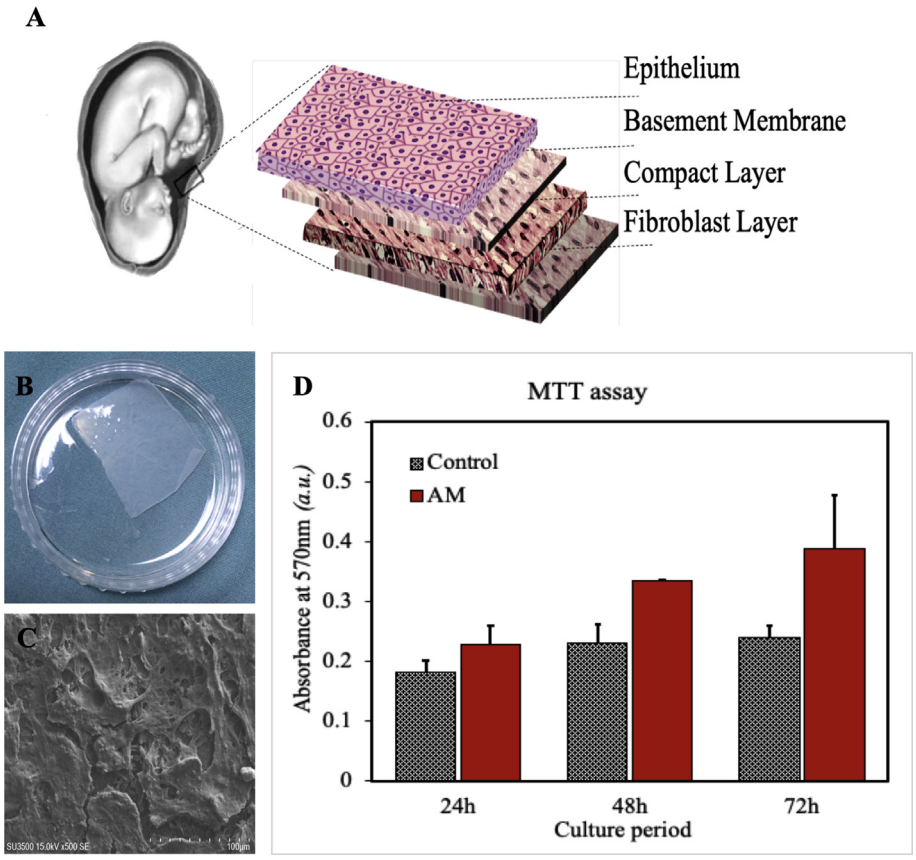


Fig. 2. (A) The schematic representation of the structure of human amniotic membrane (HAM) (B) The HAM macrostructure. (C) Scanning electron microscope (SEM) micrographs of BFSCs cells covering the HAM. (D) Cell proliferation and viability assessed by MTT in 24 h, 48 h and 72 h. Note that cell proliferation was increased from day 1 to day 3.

fibrillar collagenous structure in the extracellular matrix of HAM without any residual toxic ingredients. Observation of the BFSCs loaded HAM constructs by light microscopy at 24 h and by SEM after 3 days demonstrated that HAM was almost fully covered by BFSCs (Fig. 2B and C). Viability of the BFSCs over HAM was assessed by MTT assay at 24, 48 and 72 h. The results demonstrated significant proliferation rate of BFSCs on HAM from the 1st to the 3rd day comparing to the control (i.e., cell cultured on culture plates) (Fig. 2D).

3.3. *Clinical bone regeneration assessment*

In the present study, nine patients were studied with four in the test and five in the control group, six females and three males with the mean age of 25.87 years. All patients had received the surgery in both jaws except for one that had the surgery in her maxilla (Fig. 3). There was successful bone healing in all subjects with no evidence of inflammation, dehiscence or abscess formation and foreign-body reaction. The box plot of the vertical and horizontal bone change before and after the surgery presented in Figs. 4 and 5. The Assessment of BW2-BW1 at sites A-F demonstrated less bone formation in the vertical dimension in the control group but it was not statistically significant (4.14 mm versus 4.66 mm, $p > 0.05$). The comparison of bone formation between groups in horizontal dimension demonstrated significantly greater bone formation in the test group, 4.42 mm in the test versus 3.07 mm in the control group, $p = 0.000$).

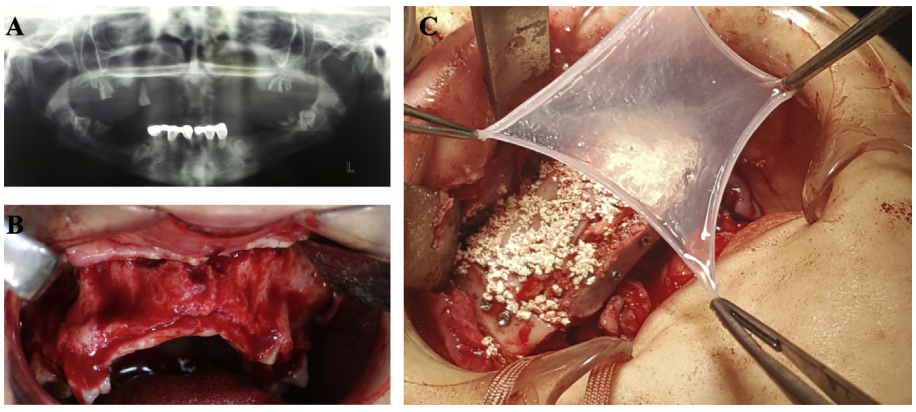
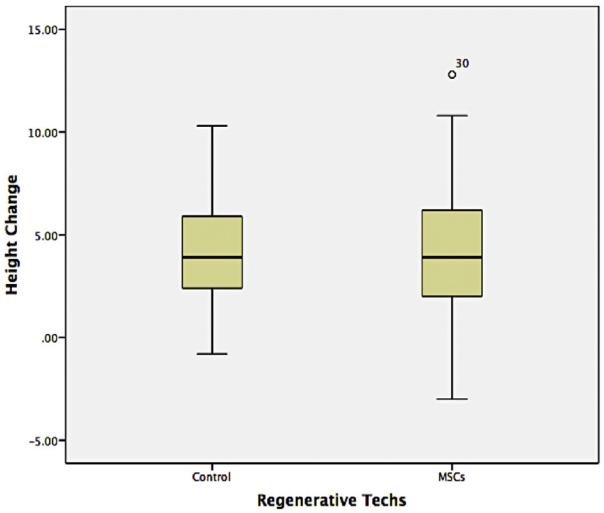


Fig. 3. (A) Radiographic evaluation of the patient (B) Exposing of the entire Bone Defect (C) placement of the bone grafts and HAM over the graft.

A



B

	BH1 (mm)	BH2 (mm)	Change(mm)
Control group			
Minimum	2.3	7.7	5.4
Maximum	14	15.3	1.3
Mean ± SD	6.58 ± 2.47	10.72 ± 1.91	4.14 ± 0.5
Test group			
Minimum	1.2	7.1	5.9
Maximum	14.9	16.8	1.9
Mean ± SD	6.27 ± 3.53	10.93 ± 2.47	4.66 ± 1.0

Fig. 4. (A) Box plot of vertical bone change before and after the surgery. Both techniques seem to yield a similar pattern although the HAM + BFSCs (MSCs) group yielded a greater range and slightly greater mean. (B) Vertical bone values preoperatively and post-operatively. BH1 = preoperative bone height, BH2 = post-operative bone height, SD = Standard deviation.

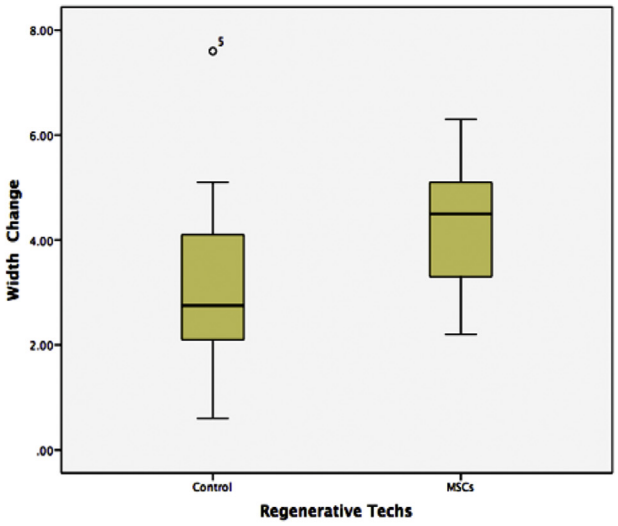
4. Discussion

The rich source of osteogenic cells in autogenous bone as well as its ideal physical structure has turned this source into the gold standard treatment method for decades (Khojasteh et al., 2012); however; its great resorption (Sbordone et al., 2012) and also the significant accompanied complications such as infection, persistent pain and nerve injury (Almaiman et al., 2013) converge to highlight the potential of tissue engineering as a promising alternative approach to eliminate the need for harvesting autogenous tissue or as an adjuvant therapy to enhance the outcomes of conventional methods. Scaffolds are one of the major modalities in tissue engineering and serve as a template for cell attachment (Jafari et al., 2017). HAM has been applied as a healing barrier in repair of various defects such as burns and ocular surgeries. Its unique properties include inherent various growth factors, anti-inflammatory properties as well as modulating angiogenesis (Niknejad et al., 2008). In the present study the authors opt to combine the regenerative properties of HAM and BFSCs to enhance bone regeneration and also to reduce bone resorption by creating a protective membrane with possible osteogenic potential.

Bone tissue engineering has been tested in various types of defects. Combination of MSCs and hydroxyapatite/β-tricalcium phosphate (HA/β-TCP) has demonstrated 41.34% bone formation in augmentation of maxillary sinus floor (Shayesteh et al., 2008). Moreover, co-application of fibrin glue with MSC loaded xenografts has demonstrated 2.09 mm bone formation around implants comparing to 1.03 mm in the groups lacking MSCs (Khojasteh et al., 2013). HA/TCP scaffolds loaded with MSCs have demonstrated 65.78% bone formation in 10 mm canine mandibular defects versus 44.9% in the HA/TCP lacking the cellular element (Jafarian et al., 2008). Treatment with combination of MSCs and scaffolds has demonstrated greater bone regeneration in critical-sized rat calvaria defects rather than scaffolds and platelet rich plasma (PRP) as 1.44 and 2.53 mm bone was formed with MSCs loaded scaffold complex comparing to 1.27 and 1.21 mm in cell-free scaffolds with PRP (Khojasteh et al., 2008). Closure of alveolar cleft with combination of allograft and MSCs resulted in 25.6% and 34.5% bone formation (Behnia et al., 2009b), while triple combination of HA/TCP, MSCs and platelet derived growth factors yielded 51.3% new bone in secondary alveolar cleft repair (Behnia et al., 2012). The present literature may highlight the potential of MSCs, scaffolds and growth factors as an adjuvant in treatment of bone defects. Protecting the onlay iliac graft with bone substitutes loaded with BFSCs in extensively resorbed jaws has demonstrated to decrease secondary bone resorption and enhance bone regeneration (Khojasteh and Sadeghi, 2016).

Owing to the healing properties of HAM and its inherent growth factors, this membrane has been studied in several defects. In gingiva scars, it yielded faster epithelialization, more organized and denser collagen fibers (Rinastiti et al., 2006), and several studies have already verified osteogenic potential of AM in vitro (Lindenmair et al., 2010; Mohr et al., 2010). Comparison of collagen with AM as a coverage for bone defects filled with Bio-Oss® demonstrated comparable results regarding mineral density however, bone tissue area in AM group was similar to normal bone and the newly formed cortical bone had greater density and cellularity comparing to the collagen group (Li et al., 2015). Treating critical-

A



B

	BW1 (mm)	BW2 (mm)	Change (mm)
Control group			
Minimum	1.7	3	1.3
Maximum	5.1	10.8	5.7
Mean ± SD	2.78 ± 0.73	5.86 ± 1.46	3.07 ± 0.73
Test group			
Minimum	1	4.2	3.2
Maximum	3.2	9.5	6.3
Mean ± SD	1.97 ± 0.59	6.39 ± 1.62	4.42 ± 1.03

Fig. 5. (A) Box plot of horizontal bone change before and after the surgery. The technique incorporating mesenchymal stem cells (MSCs) (HAM + BFSCs) demonstrates greater values and mean. (B) Horizontal bone values preoperatively and post-operatively. BW1 = preoperative bone width, BW2 = post-operative bone width, SD = Standard deviation.

sized femoral defects of mice by mixture of AM with commercial bone graft demonstrated greatest bone fill, 49.2% versus 37.8% in AM + graft and solo graft respectively however AM was not used as a membrane in this study and was delivered to the defect as a mixture with the bone graft (Starecki et al., 2014). Implanting autogenous demineralized dentin matrix (ADDM) with HAM in rabbit calvaria defect suggested that co-application of HAM with an osteoinductive material may further potentiate this membrane in bone healing (Gomes et al., 2001). Co-application of HA granules with AM in two-wall periodontal defects decreased IL-1β levels in crevicular fluid. In addition, greater bone fill, attachment gain and periodontal pocket depth healing was observed. Hence, AM was introduced as a potential membrane for guided tissue regeneration (GTR) (Kumar et al., 2015). As discussed earlier, there are several studies supporting that co-application of cell therapy with conventional bone grafting may further enhance the outcomes (Jafarian et al., 2008; Kaigler et al., 2015). This notion among the evidences on healing and osteogenic properties of AM has tempted the author to test whether combination of these elements would enhance the treatment of bone deficiencies. Our results have demonstrated that combination of AM with MSCs is more potent rather than sole use of AM as a membrane and this complex was more potent in horizontal dimension which may be explained by the challenging nature of reconstructing vertical defects. The authors assume that enhanced bone formation by HAM and BFSCs may be explained by two phenomena; negating bone resorption by this complex and or compensating it by its added osteogenic activity.

Although the current study lacked a control group (without any membrane coverage), the previous literature demonstrates 3.01 mm width gain with conventional treatment (Khojasteh and Sadeghi, 2016) which is similar to the control group in this study (3.07 mm) versus 4.42 mm in the experimental group. Besides, it should be notified that this study is also associated with several shortcomings such as lack of long-term follow up and limited number of the samples.

5. Conclusion

In conclusion, dual delivery of BFSCs with HAM may further enhance clinical bone regeneration. Future studies with emphasize on comparison of other membranes with AM and strategies to boost vertical bone height is recommended.

Author contributions

The manuscript was prepared through contributions of all authors. All authors have contributed to design, analysis and interpretation of data and drafted the manuscript. A. Khojasteh is the principle investigator.

Conflicts of interest

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcms.2019.03.030.

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