Cell Tissue Bank (2016) 17:69–83
DOI 10.1007/s10561-015-9518-5

ORIGINAL PAPER

Evaluating the bone regeneration in calvarial defect using osteoblasts differentiated from adipose-derived mesenchymal stem cells on three different scaffolds: an animal study

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Received: 30 November 2014 / Accepted: 19 June 2015 / Published online: 25 June 2015

Abstract The aim of this study was to investigate the effect of three different scaffolds on the viability and differentiation of adipose-derived mesenchymal stem cells (ADMSCs) to osteoblast for bone regeneration of calvarial defect in rabbit model. Adipose was harvested from the nape of 12 rabbits by direct surgery or hollow-tip cannula. Two standardized circular calvarial defects (case and control), 8 mm in diameter each, were created in all the animals. The animals were divided into 3 different groups. In group 1 (G1), the defect was filled with polyamide + ADMSC. In group 2, poly lactic-co-glycolic acid + ADMSC was used. In group 3, decellularized amniotic membrane + ADMSC was applied. In the control defect, the non-seeded scaffolds were applied for filling the defect. Decellularized pericardial scaffolds were used as a membrane on the scaffolds. The animals were euthanized 2, 4, and 8 weeks of operation and new bone formation was assessed by different analyses. Immunohistochemical (IHC) staining with osteopontin and osteocalcin antibodies was also performed. After 2 weeks of wound healing, minimal bone regeneration was detected in all groups. Almost complete defect closure was observed in all experimental groups after 8 weeks of operation, with the greatest defect closure in the animals treated with polyamide scaffolds as compared to biopsies obtained from control defects and other experimental groups. The maximal tensile load was higher in G1, 4 and 8 weeks postoperatively, suggesting the usefulness of polyamide + ADMSC for bone regeneration in calvarial defects. Results of the IHC staining demonstrated a significant difference between seeded and non-seeded scaffold in both short- and long-term follow-ups ($P < 0.05$). In addition, a significant difference was observed in enhancement of IHC staining of both markers in polyamide group (seeded or non-seeded) 4 and 8 weeks postoperatively in comparison with other scaffolds. It was concluded that bone regeneration in critical calvarial defect was more successful in seeded polyamide.

Keywords Adipose · Mesenchymal stem cells · Calvaria · Bone regeneration · Scaffold

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Introduction

Developments in the field of dentistry were dramatic during the last few years. Meanwhile, the application of biodegradable scaffolds, stem cells (SCs) and specific growth biomarkers like VEGF, FGF, BMP, and Emdogain has been significantly increased (Rakmanee et al. 2008). Bone regeneration in calvarial defects following trauma or congenital deformities is still a challenge for surgeons. Over the past years, the beneficial effects of tissue engineering methods on bone regeneration have been verified. It seems that the most extensive application of tissue engineering is in the area of mineralized tissue repair (Goldberg and Smith 2004).

The capability of self renewal and generating a wide range of specialized cell types are two major properties of SCs (Anderson et al. 2001). SCs have the ability to produce and differentiate to mature progeny cells which influence the cell-base therapy approaches. Mesenchymal stem cells (MSCs) that possess the capacity to differentiate into osteoblasts have been applied to evaluate bone regeneration in calvarial defects (Koob et al. 2010). Adipose tissue has been considered as a more practical and easy source for deriving an abundant supply of MSCs as compared with bone-marrow (Kim et al. 2014). In the research for alternative sources of MSCs, adipose tissues have been evaluated for their potency to repair calvarial defects. Zuk et al. (2001) described the properties of human adipose-derived mesenchymal stem cells (ADMSCs) for the first time. They also confirmed that ADMSCs are able to differentiate into adipogenic, osteogenic, chondrogenic, and myogenic lineages.

In addition, a seeded three-dimensional (3D) scaffold which is needed for appropriate cell interactions is of high importance for bone or tissue regeneration (Mendes et al. 2002). ADMSCs in combination with prefabricated scaffolds can be expeditiously applied for numerous regenerative therapies (Ge et al. 2012).

In the current study, we evaluated the behavior of ADMSCs seeded on three different seeded scaffolds including poly lactic-co-glycolic acid (PLGA), decellularized amniotic membrane (DAM), and polyamide and compared the in vivo effect of pre-seeded and non-seeded scaffolds on repair of calvarial defect.

Materials and methods

Animals

Twelve rabbits, aged 9–12 months, weighting approximately 2–3 kg were selected for this study. All the animals had healthy calvarium in clinical exam. Animal selection, management and calvarial defect preparation were conducted in accordance with the Institutional Animal Care of Tehran University of Medical Sciences. Rabbits were kept in separated cages and monitored preciously for their healthy condition. All the experimental animals were under standard laboratory diet during the study.

Decellularization procedure

Pericardium

Rabbit pericardial tissue was decellularized with 1 % Trypsin and 0.02 % ethylenediaminetetraacetic acid (EDTA) at 35 °C for 12 h. The procedure was followed by sodium dodecyl sulphate (SDS) 0.5 % for 6 h. The previous steps were conducted under continuous shaking. Finally, decellularized pericardium was washed four times for periods of 10 min with phosphate-buffered saline (PBS) in order to remove residual substances.

Amniotic membrane

Rabbit amniotic membrane was placed in distilled water at 4 °C for 2 h. Then, 0.2 % SDS was applied for the next 5 h. In the next stage, DAM was maintained in PBS containing a cocktail of antibodies.

ADMSCs isolation and culture

Fatty tissue (500 mg) was obtained from the nape of 12 rabbits under sterile condition and washed with sterile PBS. After mincing the tissues finely with a scalpel, they were placed in collagenase type 1 solution (0.1 mg/ml) for 1 h at 37 °C in a shaking water bath for digestion. In the next step, a 70-μm mesh filter (falcon) was applied for filtering the solution. Afterwards, cells were centrifuged and cultured in Dulbecco's modified Eagle's medium (DMEM), containing L-glutamine and penicillin/streptomycin. Subsequently, For the purpose of cell

attachment, the outgrown cells were incubated at 37 °C in a humidified atmosphere of 5 % CO2 and 95 % air for 36 h containing 10 % AS, fetal bovine serum (FBS) or serum-free medium + bFGF (10 ng/ml). Non-adherent cells were washed away and ADMSCs were isolated after culturing of plastic-adherent cells (ADMSCs) on the third day. The medium was changed with 3 days intervals. After the first culture, cells were placed in 0.025 % Trypsin/EDTA for 2 min in the incubator. Bone morphogenetic proteins (BMPs) consisting of osteocalsin and osteopontin were added to the subcultured cells in the third passage for better differentiation to osteoblast.

Identity of isolated stem cells

Flow cytometry analysis

Harvested cells of the third passage were incubated with CD44, CD29, CD73, CD45, CD90, and CD105 antibodies as MCSs markers. Then, the labeled cells were analyzed by flow cytometry.

Differentiation potential of ADMSCs

In order to investigate the differentiation potential of cultured cells toward osteogenic cell lineage, ADMSCs from the third passage were placed in DMEM consisting of 50 mg/ml ascorbic 2-phosphate, 10 nM Dexamethason, and 10 mM β-glycerol phosphate. At the end of this period, alizarin red staining was used to show the deposition of mineralization matrix. For staining, the cultures were first fixed by methanol for 10 min and then subjected to alizarin red solution (2 g in 100 ml water) for 2 min.

Providing seeded scaffolds

The in vitro cell-seeding process was performed in 3 passages. Cells were washed and harvested using a 0.05 % Trypsin solution. All scaffolds were cut into 1.5×1.5 cm^2 samples for cell culture. The scaffolds were sterilized using 75 % ethanol for 2 h in a 24-well plate and rinsed once in PBS and then in Hanks' buffered saline solution (HBSS) for 60 min before the cell seeding process. Sterilized scaffolds were transferred into 24-well culture plates and immersed in α-MEM overnight. At a density of 1×10^5 cells per cm^2, ADMSCs were seeded on PLGA, polyamide, and DAM using a dynamic seeding method on a rotating shaker for 6 h in 5 % carbon dioxide at 37 °C at 95 % humidity. Finally, the scaffolds were placed in a 6-well culture dish flask with culture media to allow free contact of the media with the samples.

DNA quantification

In this procedure, the cell walls/membranes are disrupted in order to isolate DNA. Lipids, proteins, and sugars are also separated from nucleic acid. This method was conducted according to the method described by Laird et al. (1991). For DNA quantification, 1 mg samples from decellularized pericardium and DAM were obtained and homogenized in a solution containing 0.25 % trypsin and 1 mM EDTA in deionized water. The homogenate was incubated with invariable stirring for 3 h at 37 °C. In the next step, the cell lysis was continued with a solution containing 2 % SDS, 5 mM EDTA, 200 mM NaCl, and 100 mM TRIS–HCl, pH 8.5 for 24 h at 55 °C. The DNA extraction was performed in isopropanol and later dissolved in a solution of 10 mM TrisHCl, 0.1 mM EDTA, pH 7.5. Spectrophotometrically at 260 nm was applied for determining the amount of DNA.

Scanning electron microscopy (SEM)

Several images with different magnifications were taken from PLGA, polyamide, DAM, and all the pre-seeded scaffolds after 8 weeks of operation and analyzed using SEM (S3500N; Hitachi High Technologies America) at voltage of 5 kV in order to determine the efficacy of our decellularization procedure in cell removal and preservation of extra cellular matrix (ECM). In order to determine the efficiency of in vitro cell seeding, pre-seeded scaffolds were placed in 2.5 % Glutaraldehyde at 4 °C for 1.5 h. Afterward, they were washed with PBS three times for periods of 30 min at 4 °C. They were then dehydrated in ethanol with concentrations of 30, 50, 70, and 90 % and processed under a critical point dryer (Autosamdri-814; Tousimis) for 15 min. In the next step, the samples were coated with gold (2 nm thick approximately) by the application of a Gatan ion beam coater.

Surgical procedure and defect treatment

Food was withheld the night before the surgery. A prophylactic antibiotic (cefazolin; 22.0 mg/kg) was

administered pre-operatively. The animals were anesthetized by intravenous injection of diazepam (0.2 mg/kg) and ketamine (6.0 mg/kg). Calvarial infiltration anesthesia was applied at the surgical sites. The animals were randomly divided into three groups. Two holes of 8 mm were made in calvarium by the application of trephine dental drills in all the animals. One of the standardized circular calvarial defects was considered as control in which the non-seeded scaffolds were applied for filling the defect. The other standardized calvarial defect was repaired using three separated methods in each group. ADMSCs (at a density of 1×10^5 cells per cm^2) were seeded on three different scaffolds. In group 1 (G1), the area of the defect was repaired with polyamide + ADMSC. In group 2 (G2), PLGA + ADMSC was used at the site of defect and in group 3 (G3), DAM + ADMSC was applied. Decellularized pericardial scaffolds were also used as a membrane on all the scaffolds (Fig. 1).

Animals received standard laboratory diet 2 weeks postoperatively. Daily intramuscular injection of Enrofloxacin 2.5 mg/kg was performed within the first 2 weeks to prevent infection. Biopsies were taken at 2, 4, and 8 weeks after the operation for investigating the regenerative capacity of the seeded and non-seeded scaffolds via further histological observations and biomechanical analysis.

Histological processing

To evaluate the effectiveness of ADMSCs in healing process, histological examination was accomplished by a pathologist who was totally blind to the current strategy. In order to achieve this objective, a specimen of 4 mm^2 of all the experimental and control samples was fixed in 10 % buffered formalin for 2–4 days, decalcified in 5 % formic acid for 6–8 weeks and dehydrated in graded ethanol. Then, the tissue blocks were embedded in paraffin for further staining with haematoxylin and eosin (H&E). For image analysis, photoshop CS3 software (Adobe Systems, Inc., Mountain View, CA, USA) and Image Pro (Image Pro Inc., Boston, MA, USA) were applied. IHMM, Ver.1, sbmu, Irani software was used for evaluation of regenerated bone and inflammation.

For determining the efficacy of different seeded and non-seeded scaffolds and identifying the regeneration of bone elements on different scaffolds, immunohistochemical (IHC) staining with osteopontin and osteocalcin antibodies was performed (Dako, Trappes, France). Photoshop 10.0 software (Adobe Systems, Inc., Mountain View, CA, USA), and Image Pro (Image Pro Inc., Boston, MA, USA) were applied for analyzing the images at each time point. SPSS 16 (Chicago, IL, USA) was used for data analysis.

Mechanical properties

Scaffolds must be able to endure intense mechanical loads in native organs. To evaluate the tensile strength, pre-seeded scaffolds of all groups were tested 4 and 8 weeks postoperatively via a tensile-test device (Zwick/Roell, Model: Hct 400/25, Germany). A constant elongation rate of 0.1 mm/s (6 mm/min) was used after clamping the samples in sample holders. Each sample was subjected to mounting uniaxial tensile testing until the emergence of tear in the tissue and disappearance of load demonstrated by the device. Parameters such as stiffness (N/m), elastic modulus and maximum force (N) were documented by the device. Eventually, the curve of strength–stress was drawn by the system in which the maximal point indicates the maximum pressure tolerance. Four samples from each group were tested, and the results were averaged.

Statistical analysis

The statistical analysis was performed using SPSS®, version 19. One-way analysis of variance (ANOVA) and independent sample *t* test were used for comparing graft particles retention and group means. Inflammatory infiltrate scores were compared between treatment and control groups by Wilcoxon's signed-rank test. *P* values less than 0.05 were considered as statistical significance.

Results

Identity of isolated stem cells

Single cell suspension of primary cultured ADMSCs was plated in 25-cm^2 culture flasks under specific culture condition to confirm the characterization of the cultured cells. The formation of approximately 25 single colonies with spindle-shaped cells authenticated the ability of ADMSCs to form adherent

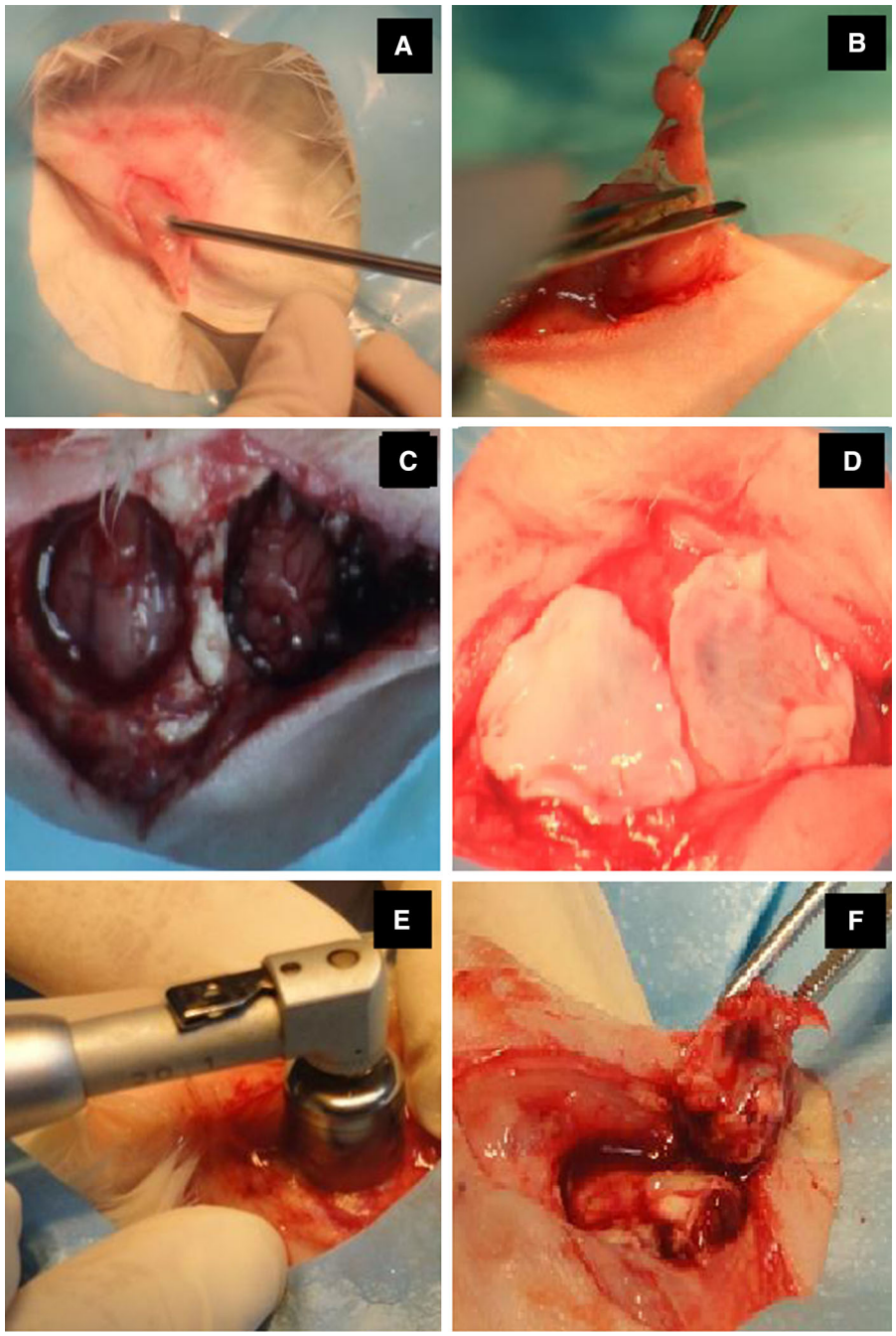

Fig. 1 Surgical technique: Adipose harvest with hollow Tip Cannula (**a**) Adipose harvest with direct surgery (**b**) Creation of two calvarial defects (8 mm) for case and control samples (**c**) The defects were repaired with different seeded and non-seeded scaffolds and covered with decellularized pericardial tissue (**d**) biopsies are taken after 2, 4, and 8 weeks of bone regeneration (**e**, **f**)

clonogenic cell clusters of Mesenchymal-like cells. Microscopic evaluation of primary ADMSCs demonstrated large flattened, long spindle-shaped, and short-shaped cells after 7 days of the initial plating. Flow cytometry outcomes demonstrated that the expression of CD44, CD29, CD73, CD45, CD90, and CD105 was

96.7, 99.5, 65.7, 99.7, 99.4, and 99.8 % respectively. The results confirmed the exhibition of MSCs properties in the majority of ADMSCs (Table 1; Fig. 2). Alizarin red staining was applied to analyze ADMSCs ability to differentiate into osteocytes that revealed mineralization in their matrix. Alizarin red staining was associated with distribution of mineralized deposits throughout the tissue culture wells (Fig. 3).

Histopathological examination

By the end of decellularization process, both pericardial tissue and amniotic membrane became whitish and translucent in appearance. The gross appearance of both tissues was maintained while DAPI staining revealed that complete cell removal was achieved (Fig. 4).

All rabbits survived the whole period of our study. Grafted scaffolds persisted at the same position without significant shrinkage, reactive or infectious changes in macroscopic view at the time of biopsy. However, microscopic evaluation of grafts revealed variable grades of regeneration in implanted scaffolds. After 2 weeks of operation, minimal bone regeneration was detected in all groups which were negligible and increased throughout the study period. Significant enhancement in bone regeneration was observed in all groups as compared to control specimens 4 weeks post operatively ($P < 0.05$). At this time, all groups formed bony bridging in areas of the defect which was more significant in polyamide + ADMSC group ($P = 0.03$). According to histopathological evaluations, new bone formation was localized to the area of the scaffold which was contiguous with the dura mater by 4 weeks of healing. This result can verify probable signaling between the underlying dura mater and ADMSCs within the scaffolds. After 8 weeks of operation, new bone was expanded into both pre-seeded and non-seeded scaffolds. In spite of the fact that bone regeneration was better in case specimens in all groups 2 months after surgery, no significant difference was detected between case and control samples. Bone regeneration was significantly enhanced in all groups in biopsies obtained 8 weeks after surgery in comparison to those taken at 4 weeks ($P < 0.05$) (Table 2) (Fig. 5). Inflammation percentage is also demonstrated in Table 3. Accordingly, inflammation was 10–30 % in all groups after 4 weeks of implantation. However, it was >10 % in all groups after 2 months. Moreover, blood vessel infiltration could be seen in the experimental bone repair area of all groups.

IHC staining was also performed by the application of antibodies against osteopontin and osteocalcin. IHC staining of both markers revealed a significant difference between seeded and non-seeded scaffold in both short- and long-term follow-ups ($P < 0.05$). Nevertheless, No significant difference was observed in biopsies taken after 4 and 8 weeks in none of the groups ($P > 0.05$). However, a significant difference was observed in enhancement of IHC staining of both markers in polyamide group (with or without ADMSCs) after 4 and 8 weeks of operation in comparison with other scaffolds (Figs. 6, 7; Table 3).

Table 1 Data are means (% of normal bone tissue)

P value[a]	8 weeks		4 weeks		Group
	Control	Case	Control	Case	
Bone regeneration (%)					
0.01	42.50	62.50	9.35	21.75	Polyamide + ADMSCs
0.03	29.84	44.68	4.65	11.64	Polyglycolic acid + ADMSC
0.04	17.31	22.54	2.47	5.42	Amniotic membrane + ADMSC

$P < 0.05$ is considered statistically significant. Bone regeneration was significantly enhanced in all groups as compared to control specimens 4 and 8 weeks post operatively

Bone regeneration was better in case specimens in all groups, 2 months after surgery. However, no significant difference was detected ($P > 0.05$)

[a] Bone regeneration was significantly enhanced in all groups in biopsies obtained 8 weeks after surgery in comparison to those taken at 4 weeks

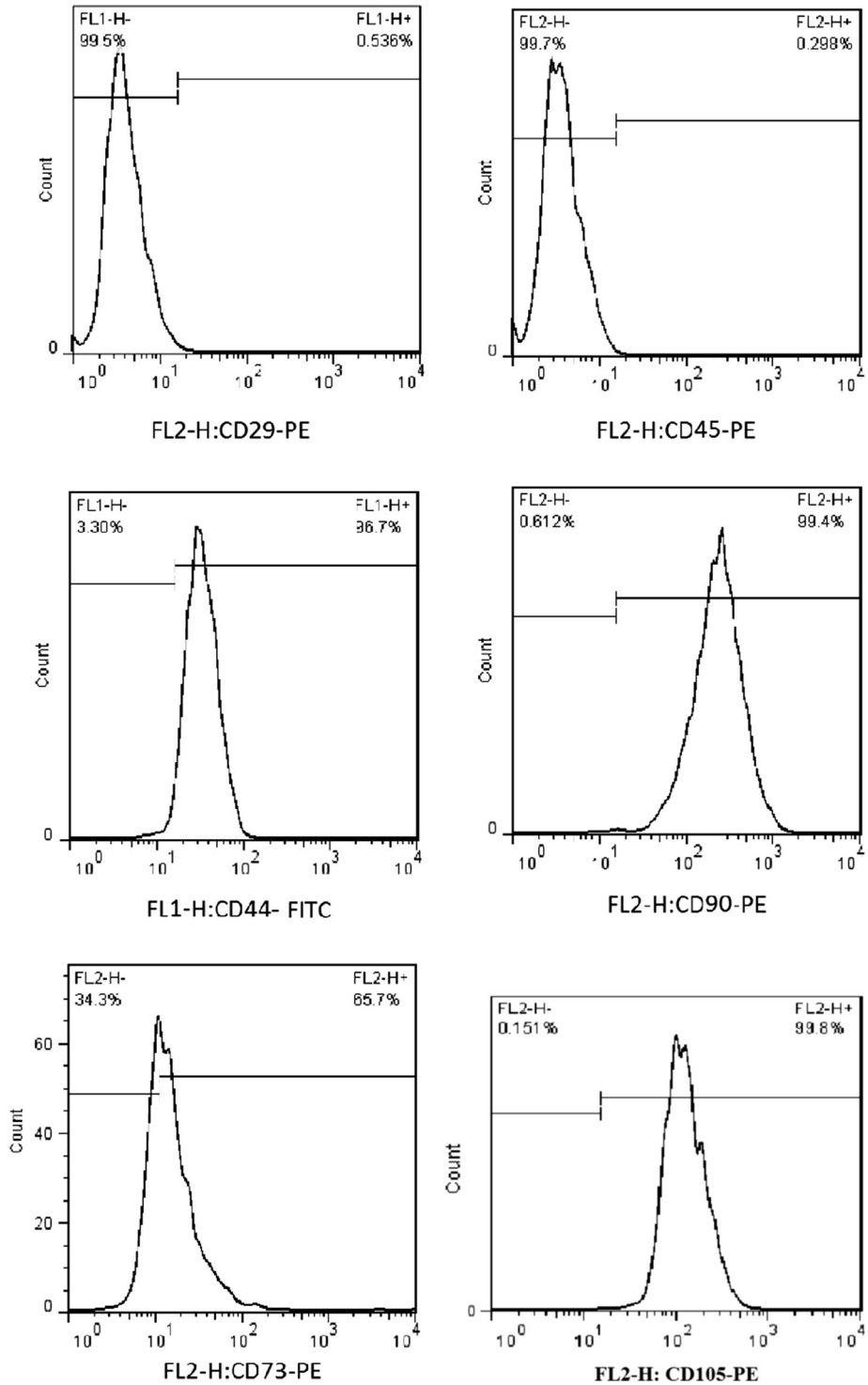

Fig. 2 Flow cytometry: Expression of CD44, CD29, CD73, CD45, CD90, and CD105 were evaluated to show that cultured cells are ADMSCs

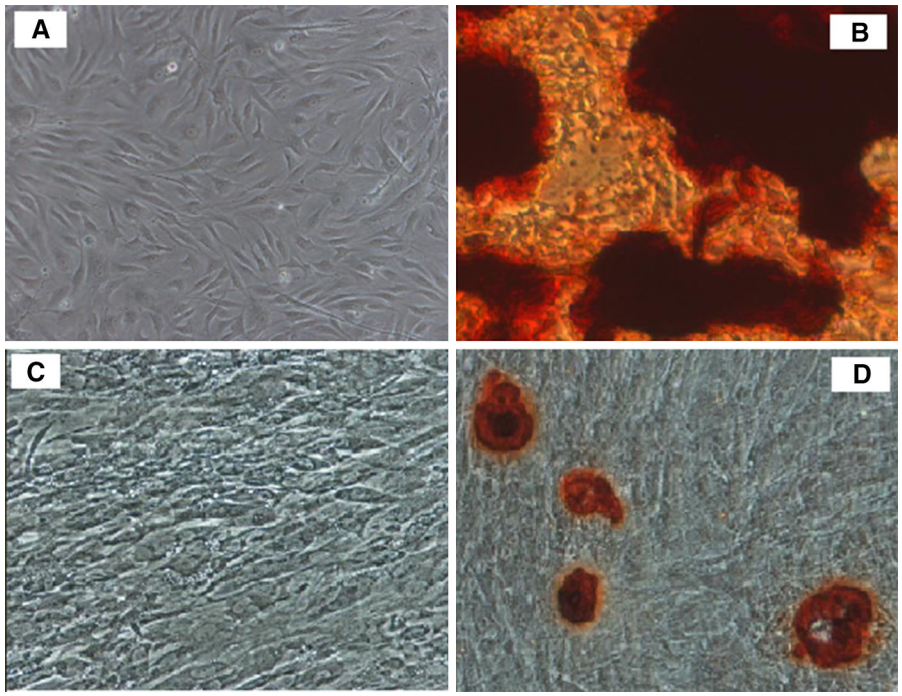

Fig. 3 Undifferentiated adipose cells (**a**) Alizarin-red staining of undifferentiated cells (**b**) Differentiated ADMSC after being cultured in specific culture media (**c**) Alizarin-red staining of differentiated cells to osteogenic cell lineage (**d**)

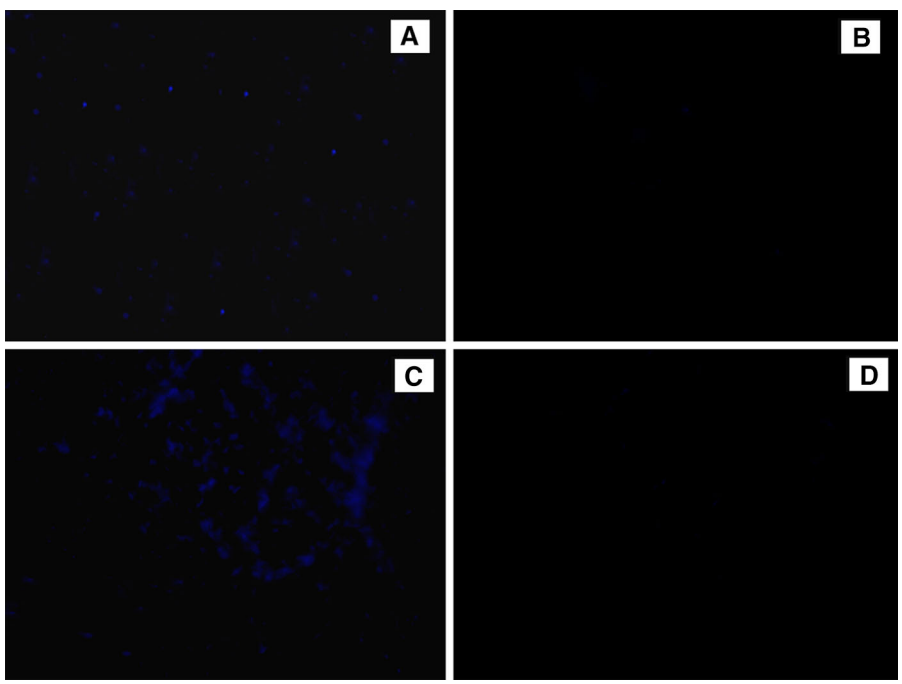

Fig. 4 DAPI staining: Natural amniotic membrane (**a**) DAM (**b**) Natural Pericardium (**c**) Decellularized pericardial tissue (**d**)

Table 2 Inflammation was similar in all case and control samples in short-term follow-up (10–30 %)

8 weeks		4 weeks		Group
Control	Case	Control	Case	
Inflammation (%)				
<10	<10	10–30	10–30	Polyamide + ADMSC
<10	<10	10–30	10–30	Polyglycolic acid + ADMSC
<10	<10	10–30	10–30	Amniotic membrane + ADMSC

The percentage of inflammation decreased to 10 % in all experimental groups without any significant difference

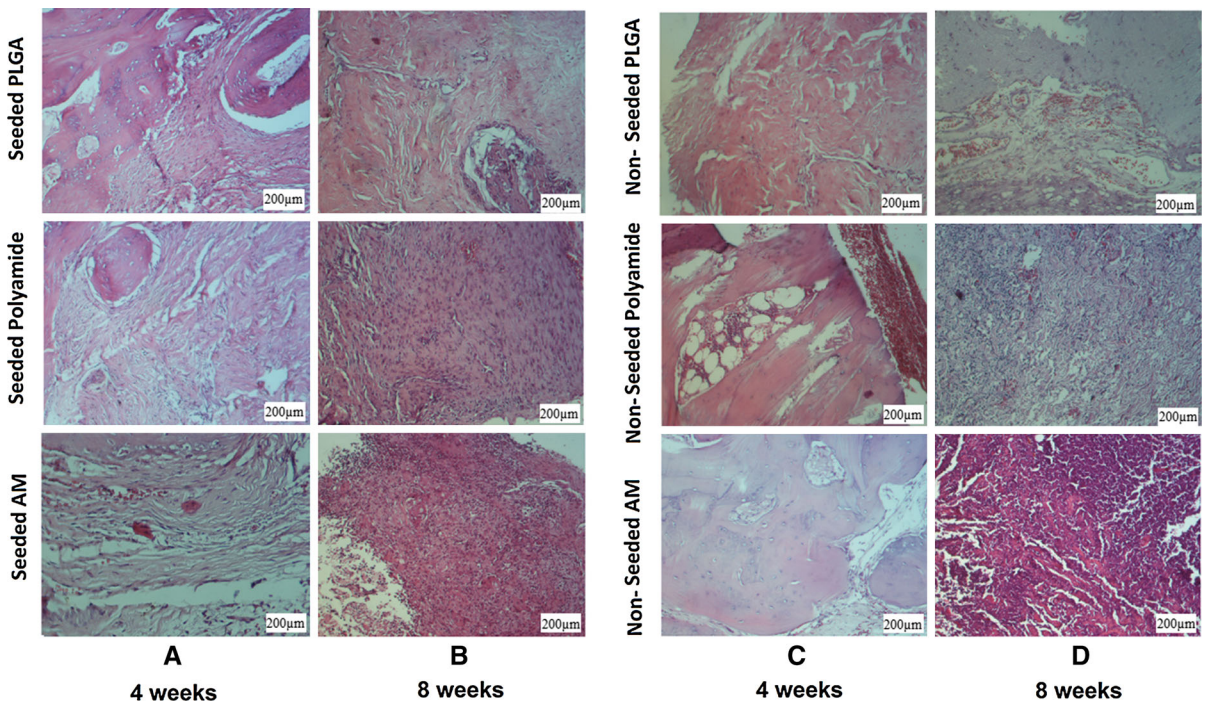

Fig. 5 Histopathological examination: H&E staining of different seeded scaffolds after 4 weeks of implantation (**a**) case samples of different scaffolds 8 weeks postoperatively (**b**) H&E staining of different non-seeded scaffolds 1 month postoperatively (**c**) control specimens of different scaffolds 2 months after surgery (**d**)

Scanning electron microscopy

SEM revealed that cells were entirely removed from DAM without any detectable disarrangement of the matrix or collagen degradation. PLGA and polyamide were examined by SEM the result of which revealed well-organized porous that were satisfactory for cell seeding. The results of SEM after 8 weeks of implantation showed well-organized cell-seeded scaffolds with more seeded cells (Fig. 8).

Mechanical properties

No significant difference was detected in the mechanical properties of the matrices in G1 compared with the properties of the native samples after 8 weeks of operation. In fact, the maximal load in both native and experimental samples of G1 was nearly the same, suggesting its usefulness for bone regeneration in calvarial defects. The cell-seeded scaffolds in G2 showed biophysical properties that were similar to

Table 3 Data are means (% of normal calvarial bone tissue)

		Osteocalcin		Osteopontin	
		4 Weeks	8 Weeks	4 Weeks	8 Weeks
PLGA	Seeded	115	121.25	132.25	140
	Non-seeded	54.5	61.75	70.5	81.75
Polyamide	Seeded	148.5	173.75	162.25	174.75
	Non-Seeded	68.25	76	96.75	104.5
AM	Seeded	104.25	113.5	124.5	131
	Non-Seeded	38.75	42.75	53.25	62.5

$P < 0.05$ is considered statistically significant. In the seeded groups, all markers revealed significant enhancement in biopsies obtained 4 and 8 weeks after surgery in comparison to the non-seeded groups ($P < 0.05$). A significant difference was also observed between the polyamide and other scaffolds (PLGA and AM) between 4 and 8 weeks of operation ($P < 0.05$)

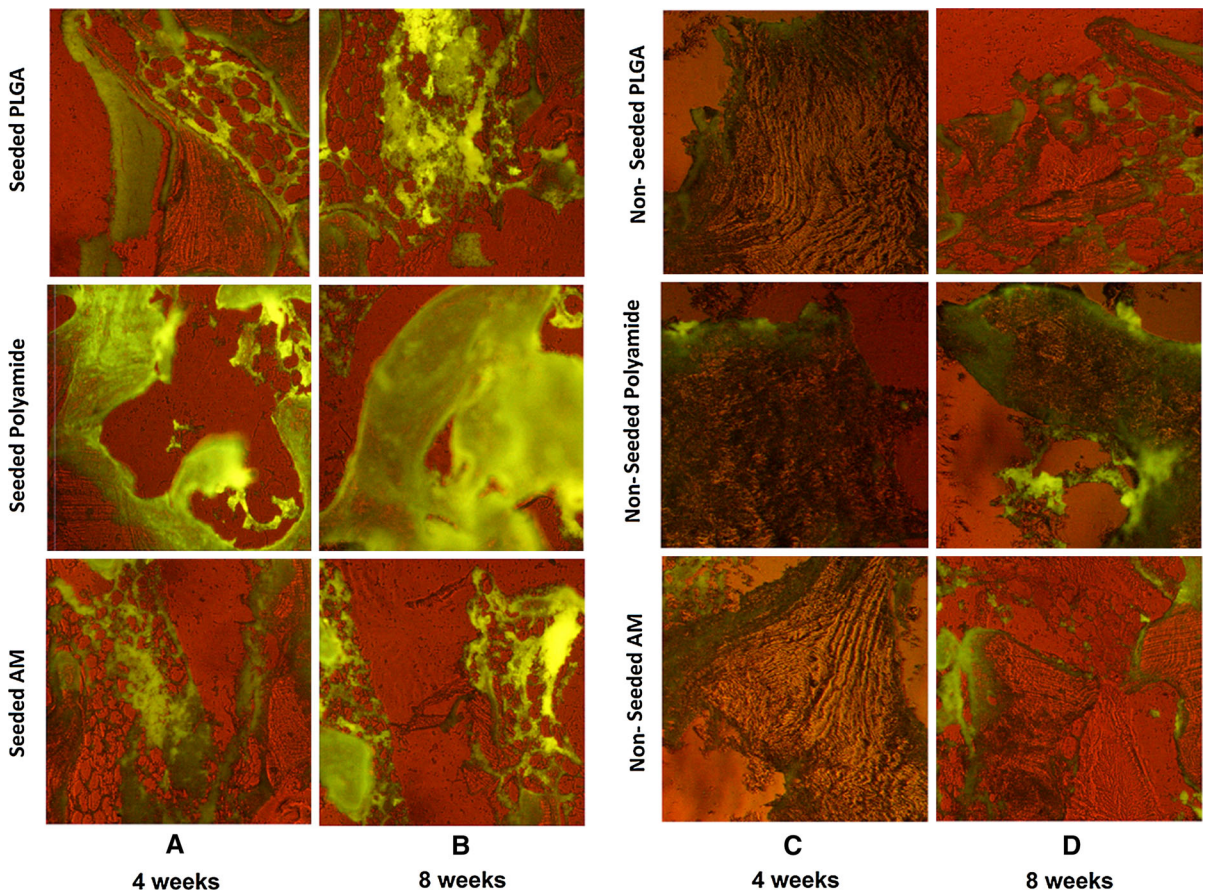

Fig. 6 IHC staining with osteocalcin: Different seeded scaffolds after 4 weeks of implantation (**a**) case samples of different scaffolds 8 weeks postoperatively (**b**) different non-seeded scaffolds 1 month postoperatively (**c**) control specimens of different scaffolds 2 months after surgery (**d**)

those of native tissues but the tensile strength of these matrices were not as well as samples in G1. The case samples in G3 had biophysical properties that were the least similar to those of the native samples. However, all the samples demonstrated improvement in mechanical properties after 8 weeks of operation, which was more significant in G1 compared to normal sample (Fig. 9).

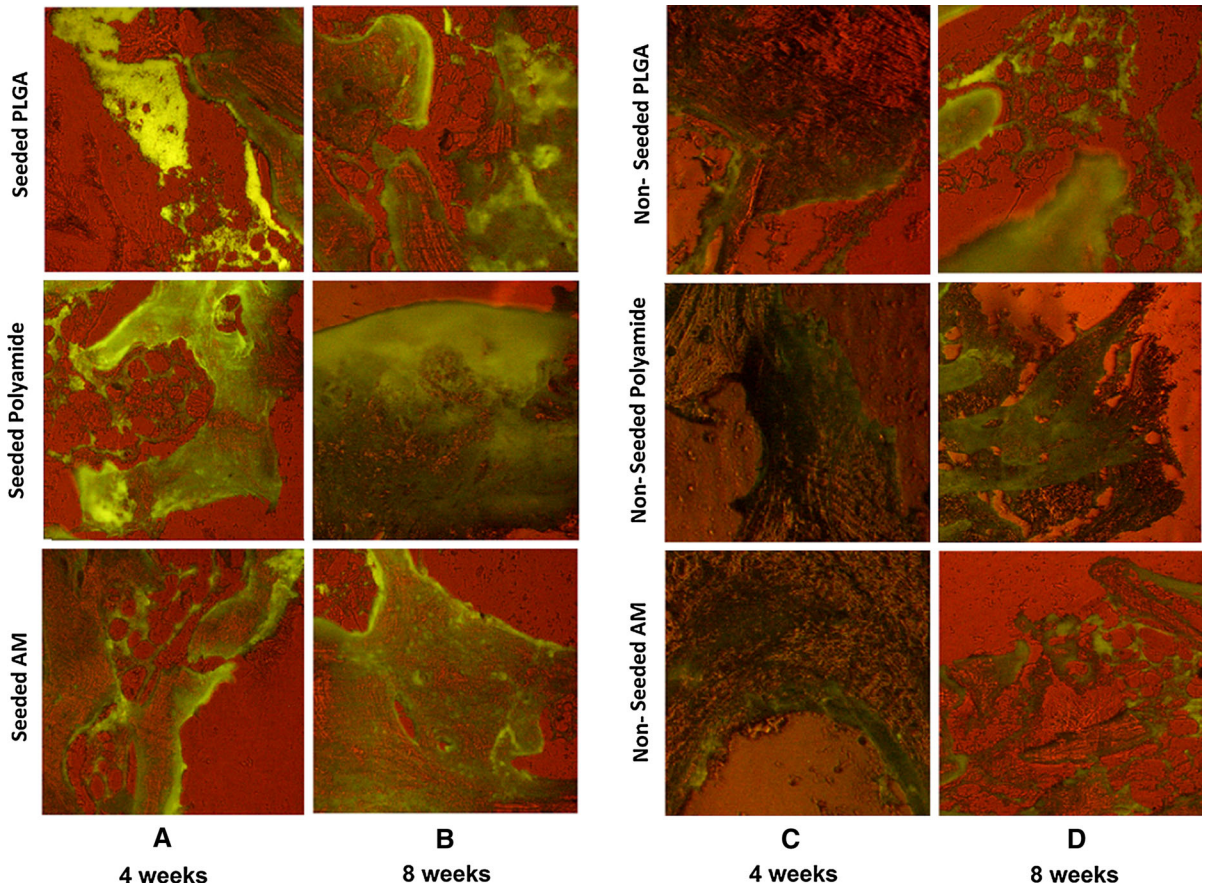

Fig. 7 IHC staining with osteopontin: Different seeded scaffolds after 4 weeks of implantation (**a**) case samples of different scaffolds 8 weeks postoperatively (**b**) different non-seeded scaffolds 1 month postoperatively (**c**) control specimens of different scaffolds 2 months after surgery (**d**)

DNA quantification

DNA quantification was less than 5 % in decellularized pericardium and DAM compared to control samples. DNA quantification demonstrated 1.4 and 1.2 ng dsDNA per mg dry weight of ECM, in decellularized pericardium and DAM, respectively. These findings demonstrated the efficacy of decellularization process in removal of cell components as well as ECM preservation that warranty its potentials in effective in vivo studies with minimal adverse host reaction.

Discussion

The aim of the current study was to evaluate the potential of three different scaffolds seeded with ADMSCs to repair calvarial defects and assess the wound healing process and bone regeneration in an animal model. In this experimental study, all the seeded and non-seeded scaffolds had the capability of bone regeneration in the calvarial defect after 8 weeks of follow-up. However, new bone formation was more significant in polyamide + ADMSC group.

Developmental anomalies, acute and chronic pathological processes, and trauma may cause patients to lose their natural calvarial bone. In spite of the fact that no intervention is needed in small bone defects and they can repair spontaneously, reconstructive surgery is necessary in extensive bone damages. The success in bone regeneration depends on the morphology of the defect and the graft material (Lupovici 2009). In the wake of such phenomena, we tried to determine the best method to overcome this noticeable complication and recreate natural calvarial bone

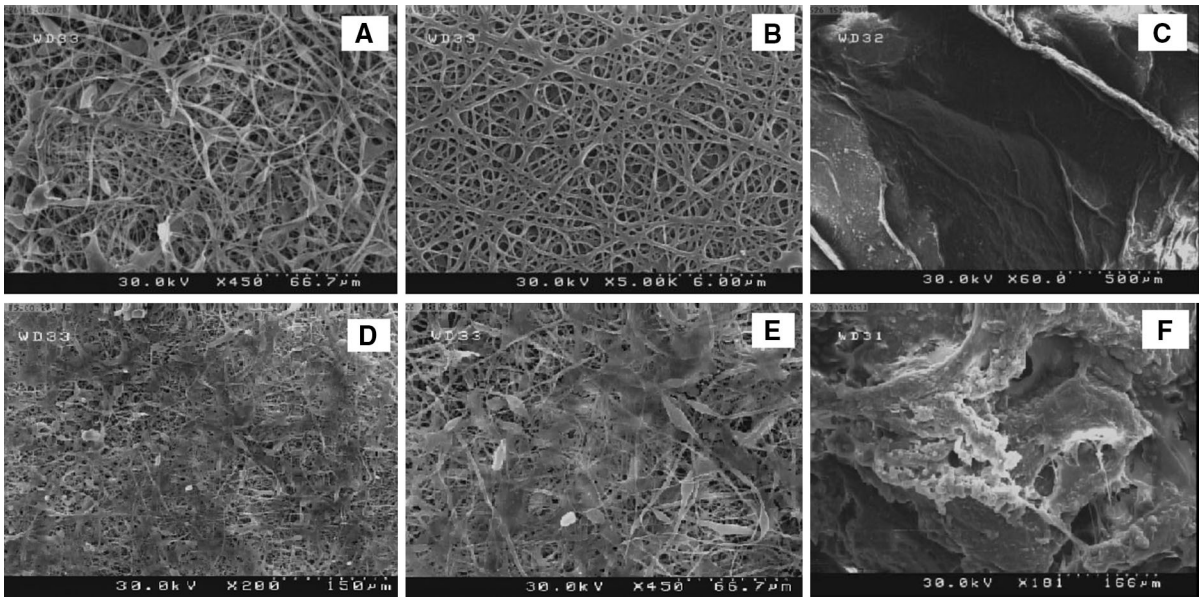

Fig. 8 Scanning electron micrograph: PLGA (**a**), polyamide (**b**) and DAM (**c**) after 4 weeks of transplantation. PLGA (**d**), polyamide (**e**), and DAM after 8 weeks of transplantation

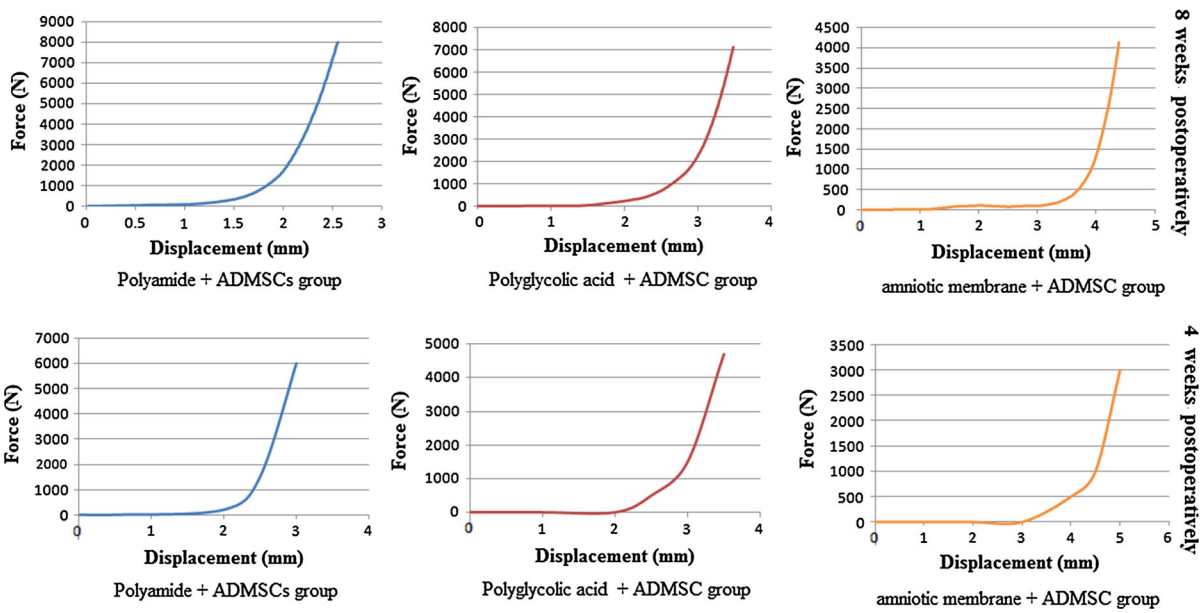

Fig. 9 Biomechanical properties of natural tissue and different scaffolds 4 and 8 weeks postoperatively

function. Regenerative procedures for obtaining a feasible and newly formed calvarial bone still remain challenging. However, the application of pre-seeded or even non pre-seeded scaffolds may be the safest strategy for bone engineering and tissue regeneration. Considering the fact that a critical size defect model is crucial for evaluating biomaterials in bone defect, a standardized rabbit calvarial defect was selected for this experiment.

The therapeutic advantages of delivering cells in biodegradable scaffolds have been demonstrated in different aspects of tissue engineering researches (Lee

et al. 2001; Cowan et al. 2004; Kajbafzadeh et al. 2014a, b). As MSCs are capable in tissue regeneration and immunosuppressive properties, they have been considered as an excellent treatment for chronic inflammation and tissue defects (Parekkadan and Milwid 2010). Due to the fact that patients with calvarial defect may confront with several complications associated with gene therapy approaches (Lee et al. 2001; Cowan et al. 2004), using pre-seeded scaffolds with accessible source of osteogenic cells that do not need genetic manipulation may pave the road to repair calvarial defects. So, in the current study ADMSCs were avoided form genetic manipulation. In addition, in the current study the use of autogenous ADMSCs prevented many of the current limitations associated with bone grafts. Unavailability and risk of pathogen transmission are among the limitations of the autologous and allogenic bone grafts, respectively (Zomorodian and Baghaban Eslaminejad 2012). MSCs can be considered as substitute for the mentioned grafts. ADMSCs have the ability to differentiate into numerous functional SMCs and osteogenic cells which may be useful in calvarial bone regenerative medicine. ADMSCs have the ability to proliferate rapidly in vitro in comparison with bone-marrow stem cells which is important for clinical application. One of the benefits of the current study is the application of ADMSCs with better immuno-compatibility, isolation, and expansion compared to bone marrow-derived mesenchymal stem cells (Bourin et al. 2013; Kim et al. 2014). So, we tried to investigate the compatibility of ADMSCs as a cell source in treating calvarial defects.

It has been also demonstrated that bone regeneration and cell proliferation in lost bone tissue can be enhanced by the application of tissue engineered scaffolds (Yun et al. 2012). PLGA has been considered as a popular polymer in the tissue engineering field. Tissue compatibility and reproducible mechanical properties are among the noticeable properties of PLGA which are necessary especially for bone regeneration (Boland et al. 2001). Architectural support, ease of cell inoculation without leakage and stratification of the MSCs are other characteristics of the PLGA (Uematsu et al. 2005). Having the top and bottom pores is one of the characteristic of PLGA. It should be also mentioned that top and bottom pores are efficient for raising the efficiency of cell seeding and holding the seeded cells in this scaffold, respectively. In one study, three-dimensional PLGA scaffolds were seeded with MSCs and transplanted in the created defects of the knee joints of rabbits. The histological results after 12 weeks of transplantation demonstrated hyaline-like cartilage in the defects. In accordance with the results of our study, they concluded that the structure of the PLGA scaffold provided architectural support and satisfactory induction of chondrogenesis in animal model (Uematsu et al. 2005). In another study, the effect of PLGA alone, PLGA seeded with differentiated ADMSCs, and PLGA seeded with undifferentiated ADMSCs was assessed in bone regeneration by implanting the scaffolds in a critical nude rat calvarial defect. The results demonstrated that PLGA scaffolds seeded with osteogenically differentiated ADMSCs obtained better results regarding bone regeneration (Yoon et al. 2007).

Polyamide has a composition and structure very close to natural bone collagenous proteins and therefore has been considered as an ideal scaffold for repair of bone defects and biomaterial application (Wang et al. 2007). The outcome of the current study demonstrated that in the calvarial defect treated with polyamide + ADMSCs, the evidence of osteogenesis and enhanced biological activity were observed. This scaffold was superior to other applied scaffolds in both long and short-term follow-up.

However, other natural decellularized matrices which have an appropriate histological and biophysical property can be applied for the purpose of calvarial defect regeneration. Amniotic membrane contains collagen type I and this instinctive property makes it ideal for tissue regeneration procedures. It should be also mentioned that the tensile strength and formation of the bone is due to this type of collagen. To the best of our knowledge this is the first study in which DAM has been used to regenerate calvarial defect.

Due to desirable mechanical integrity, biocompatibility, and osteogenesis characteristics of PLGA, polyamide, and DAM they may have the potential to be applied in reconstructive and maxillofacial surgery. By the fixation of the applied scaffolds onto the defects, we tried to optimize architectural compatibility.

The use of a barrier membrane is crucial for satisfactory osteoinductive regeneration. Pericardium is considered as a barrier membrane with excellent biocompatibility which has been widely applied for

reconstructing the bone defect adjacent to the implants. However, few clinical studies have been performed regarding this procedure (Ahn et al. 2012). In one study, it has been demonstrated that bone graft with pericardium membrane can facilitate bone regeneration by inhibiting connective tissue invasion (Ahn et al. 2012). The results of the current study also demonstrated that decellularized pericardial tissue used as a membrane on the scaffolds, plays a crucial role in enhancing bone regeneration.

Defect type plays a crucial role in avoiding the epithelium migration and holding the implanted scaffolds in place of defect (Suaid et al. 2011). In the study of Caplanis et al., no histological effects on bone activation or regeneration attachment were found following the application of demineralized freeze-dried bone allografts in maxillary canine defects after 4 weeks. Additionally, no inflammatory cells or resorption of the implants were noticed (Caplanis et al. 1998). Findings of the current study demonstrated that by the application of polyamide + ADMSCs in area of defect, a better outcome can be achieved regarding the extension of calvarial bone regeneration. In comparison, the cell-engineered scaffolds of all groups demonstrated satisfactory biocompatibility, faster and more effective osteogenesis at the defect area compared to pure scaffolds. However, in the amniotic membrane + ADMSC group, mild inflammatory reaction was recognized. The results of this study showed that polyamide had more satisfactory results on bone generation of calvarial defect.

In the current study, we rendered an animal model to evaluate the potential of bone regeneration by the creation of calvarial bone defects. One the benefits of this study are the application of a stable carrier scaffold which is necessary for cell attachment and suitable for clinical settings. This scaffold did not show any remarkable inflammation during the healing period. However, some potential limitations should be underlined in the current study. It would be better to track ADMSCs with staining protocols before seeding the cells on the scaffolds to evaluate the role of seeded cells in repairing the calvarial defect. Moreover, more functional evaluation should be performed to ensure adequate functional property of the regenerated bone. Additionally, more long-term investigations are required to assess the feasibility and safety of this procedure, enrich our understanding of pre-seeded ADMSCs on scaffolds, and set up foundations for utilization of ADMSCs in repair of calvarial defect, before clinical use.

Conclusion

The outcomes of this preliminary report demonstrated that polyamide Nano Scaffold seeded with ADMSCs can be successfully used in repairing calvarial defects. The histological findings of this type of repair revealed the development of well-vascularized and newly formed bone. While the osteoinductivity of ADMSCs has been authenticated via in vitro examinations, further trials are recommended to support the clinical relevance.

Acknowledgments We highly appreciate Dr. Laleh Nikfarjam at the Pediatric Urology Research Center for collaborating in cell culture section of this experimental study. Special thanks to associated professor Masoud Soleimani at the Department of Hematology, School of Medical Science, Tarbiat Modares University for performing the IHC staining of the current study.

Compliance with Ethical Standards

Conflict of interest None of the authors has direct or indirect commercial financial incentive associating with publishing the article and does not have any conflict of interest, and will sign the Disclosing Form.

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