Biological Optimization of Cortical Bone Allografts: A Study on the Effects of Mesenchymal Stem Cells and Partial Demineralization and Laser Perforation

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Received: 09 Apr. 2016; Received in revised form: 15 Dec. 2016; Accepted: 27 Mar. 2017

Abstract

Background: Despite promising results have shown by osteogenic cell-based demineralized bone matrix composites, they need to be optimized for grafts that act as structural frameworks in load-bearing defects. The purpose of this experiment is to determine the effect of bone marrow mesenchymal stem cells seeding on partially demineralized laser-perforated (DLP) structural allografts that have been implanted in critical femoral defects.

Methods: Thirty-two Wistar rats were divided into four groups according to the type of structural bone allograft; the first: partially demineralized only (Donly), the second: partially demineralized stem cell seeded (DST), the third: partially DLP, and the fourth: partially demineralized laser-perforated and stem cell seeded (DLPST). Trans-cortical holes were achieved in four rows of three holes approximated cylindrical holes 0.5 mm in diameter, with centers 2.5 mm apart. P3 mesenchymal stromal cells (MSCs) were used for graft seeding. Histologic and histomorphometric analysis was performed at 12 weeks.

Results: DLP grafts had the highest woven bone formation, where most parts of laser pores were completely healed by woven bone. DST and DLPST grafts surfaces had extra vessel-ingrowth-like porosities. Furthermore, in the DLPST grafts, a distinct bone formation at the interfaces was noted.

Conclusions: This study indicated that surface changes induced by laser perforation, accelerated angiogenesis induction by MSCs, which resulted in endochondral bone formation at the interface. Despite non-optimal results, stem cells showed a tendency to improve osteochondrogenesis, and the process might have improved if they could have been supplemented with the proper stipulations.

Citation: Mirghasemi SA, Sadeghi MS, Rahimi-Gabaran N, Baghban-Eslaminejad M. **Biological Optimization of Cortical Bone Allografts: A Study on the Effects of Mesenchymal Stem Cells and Partial Demineralization and Laser Perforation.** *Acad J Surg*, 2017; 4(2): 31-6.

Keywords: Allografts; Bone demineralization technique; Laser; Mesenchymal stem cell

Introduction

Critical-sized bone defects are highly prevalent, and clinically, over 2.2 million bone graft surgeries are performed in the world annually, often resulting from skeletal defects, traumatic injuries, tumor resections and infections, they are a continuous challenge for orthopedic surgeons (1-3). Therefore, bone graft transplantation is utilized as an effective treatment for healing large defects in orthopedic reconstruction procedures (4). Consequently, autograft, allograft, or synthetic bone substitute can be used for reconstruction of bone defects (2). Autologous bone grafts considering their better osteogenic, osteoinductive, and osteoconductive properties, as well as containing live cells and growth factors and no immunologic rejection, are the gold standard for stimulating bone formation in large defects (5-9). However, these grafts have some disadvantages including donor morbidity, limited availability, risk of wound infection, bleeding, hematoma, long anesthesia time, and increasing operation time, and chronic pain (6,8,9). Thus, allografts are known as a clinical gold standard in treating critical-sized defects due to effective and reliable techniques for bone processing and detection of infectious diseases (1). Correspondingly, allografts have some advantages to autografts including unlimited volume with similar quality, and without donor morbidity, and pain (1,8). However, allografts have also some limitations including possibility of disease transmission from donor to recipient, immunologic reactions, fractures, infections, non-unions and lack of appropriate structural allografts osteogenesis, angiogenesis, and remodeling. These

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deficiencies have resulted in 60% fail after 10 years of implantation (1,4,6,7,10,11).

The role of mesenchymal stromal cells (MSCs) in tissue restoration has been studied for decades and shows that these multipotent cells have the potential to becoming different progenitor cells including osteoblasts, chondrocytes, adipocytes, tenocytes, myoblasts, and stromal bone marrow cells (5,12,13). These cells are present in bone marrow, endosteum, trabecular bone, periosteum, and less in peripheral blood circulation (5,14). Recently using these cells and osteoprogenitors together with an appropriate scaffold carrier has reached high popularity, so that when these cells are transferred to the site of bone injury, they will turn into osteoblast, chondroblast and fibroblast cell rows and participate in the production of bone and cartilage tissues (14,15). Tissue engineering provides these conditions by bone marrow cells seeding on scaffold or matrix (16). Therefore, to achieve this state, difference bound matrix (DBM) due to the close relation in structure and performance with autologous bone is a suitable choice for bone tissue engineering scaffolds (3).

Considering that drill holes in the grafts are proposed as a way for bone ingrowth improvement (17), and in spite the fact that to the best of our knowledge, no specific study has been conducted about partially demineralized laser-perforated (DLP) stem cell seeded grafts, this study attempts to examine the effect of stem cells on allografts.

Materials and Methods

hirty-two male Wistar rats weighing about 150 g (approved by the Institutional Review Committee of Royan Institute) were used in this study.

Preparation of allografts: The bone allografts were 1 cm from long midshaft femoral segments of 300 g Wistar rats. The attached tissues of allografts were stripped using surgical blades, and bone marrow was removed by intramedullary irrigation by normal saline. Transcortical holes were achieved by an Er:YAG laser (Schwartz Electro-Optics, Concord, MA, USA) at a wavelength of 2.94 µm in four rows of three holes (total 12 holes) approximated cylindrical holes 0.5 mm in diameter, with centers 2.5 mm apart. Then, allografts were made acellular by immersing in 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA) and demineralized partially with 1 M HCl for 15 minutes. The allografts were fixed in 2.5% glutaraldehyde solution at 4° C for 24 hours and then washed with phosphate-buffered saline. The samples were then dehydrated sequentially with increasing concentrations of ethanol (30%, 50%, 80%, and 100%) and visualized with an electron microscope (Zeiss, DSM 940A, Germany). Grafts were stored at −80° C until surgery.

Extraction of bone marrow and culturing stem cells: Approximately 150 µl of bone marrow was sampled from Wistar rats and mixed with 5 ml Dulbecco's modified Eagle's medium (DMEM; Ginco, Germany) in a test tube and then centrifuged at a rate of 1200 rpm for 5 minutes. The supernatant was discarded, and the cell pellet at the bottom of the test tube (that suspended in DMEM) plated in 75 cm^2 culture flasks that contained 15% fetal bovine serum, 100 IU/ml penicillin, and 100 IU/ml streptomycin (all from Gibco, Germany). The cultures were further incubated in an atmosphere of 5% CO_2 at 37° C. From this point on, every 3rd day since culture initiation, we changed the medium to remove non-adherent cells until a confluence of 70-80% was achieved. This was followed by detachment of the cells with 0.05% trypsin/EDTA and then a subculture at a ratio of 1:3 was created. The process was repeated until P3 obtained the sufficient cells to either run the *in vitro* tests and/or be used in the augmentation of grafts (18).

Preparation of stem cell-enriched allografts: In this step, P3 MSCs were detached from culture plate surfaces via trypsin/EDTA and then loaded on the allografts. For this purpose, approximately 0.5 ml of collagen gel (Vitrogel, Inamed, CA, USA) was mixed with 0.5 ml of DMEM, and the pH was adjusted to 7.4 by dropwise addition of 1N NaOH. About 2×10^6 P3 MSCs were then suspended homogeneously in 1 ml of the gel. The medullary cavity of the allograft was packed with the MSC-containing gel and applied to the cortical surface and graft-host interfaces.

Surgical procedures: In this step, the rats were anesthetized via intraperitoneal injection with a combination of ketamine (60 mg/kg body weight) and xylazine (4 mg/kg body weight) and positioned anterolaterally. The skin of the right femoral region was shaved and scrubbed with betadine (povidone-iodine). A 3 cm long full thickness incision was made in the midshaft of the femur and freed of surrounding tissue. Then under saline irrigation, a critical central defect of 10 mm was created with the use of a rotary burr. Graft implantation was fixed by 1.5 mm thick intramedullary nails. Finally, the rats were kept under similar conditions and daily controls for 12 weeks and then killed.

Histologic evaluation: Retrieved grafts along with adjacent portions of femoral bones were immediately fixed in 10% formalin for 48 hours. Then, specimens were placed in EDTA until they reached a suitable decalcification, and after that, they were dehydrated in a graded series of ethanol baths and then embedded in paraffin blocks. From the blocks of every specimen, a series of longitudinal sections of 5 µm thickness were obtained and stained with H and E to prepare for microscopic evaluation. The grafts were examined for graft incorporation and new bone formation at the interface level.

Histomorphometric analysis: Quantitative analyses were performed on photomicrographs acquired by a light microscope (BX71, Olympus) using Adobe

Photoshop CS5.1 and Image-Pro Plus (V6.0, Media Cybernetics Inc., USA). We measured the percent of the area filled by each of the parameters on sequential longitudinal sections as per the literature (10,17,19). For each graft, we determined the percentage area of graft bone incorporated either by new woven bone, resorption lacunae, cartilaginous tissue, graft remnants, endochondral ossification, or vessel in growth in the form of porosities that differed from resorption lacunae on intact graft surface. Similar parameters of woven bone or osteochondral tissue and angiogenesis were used to determine the percentage area of gap filled at the interface between the host and allograft termini.

Study design and statistical analysis: The rats were divided into four groups of eight according to the type of structural bone allograft. The first: partially demineralized only (Donly), the second: partially demineralized stem cell seeded (DST), the third: partially DLP, and the fourth: partially demineralized laser-perforated and stem cell seeded (DLPST). One-way analysis of variance (ANOVA) was used to compare statistically significant different groups using IBM SPSS Statistics (version 20, IBM Corporation, Armonk, NY, USA). Results were considered significant at $p < 0.050$. Tukey's *post hoc* was run for multiple comparisons to determine the differences if comparisons were significant.

Results

At 12 weeks, Donly grafts cortex incorporation had apparently higher resorption lacunae than woven bone formation, which predominantly started at the periosteal surface near junction sites (Figure1a and b). Replaced defects at the interface were occupied by alternative woven bone trabeculae with scants of cartilage. Correspondingly, higher degrees of graft healing between woven bone and either graft or host bone were observed (Figure 1c). Endochondral callus formation was also evident in this specific group (Figure 1d).

Similar to Donly group, DLP allografts had various degrees of graft incorporation and healing. Periosteal bone callus width was comparatively low, and we did not observe any endochondral callus as in the previous group (Figure 2a). Healing at the junction was more complete in this group, and a wider area was in contact with host and graft surfaces (Figure 2b). In this group, higher degree of bone formation was seen despite less resorption lacunae than Donly grafts. In most parts, laser pores were completely healed by woven bone, which can be the reason of upper woven bone formation at incorporation in these grafts (Figure 2c).

Similar to the Donly and DLP groups, DST allograft surfaces had different degrees of resorption and bone ingrowth (Figure 2d). They had a distinctly higher number of vascular ingrowth porosities throughout the allograft surfaces (Figure 2e).

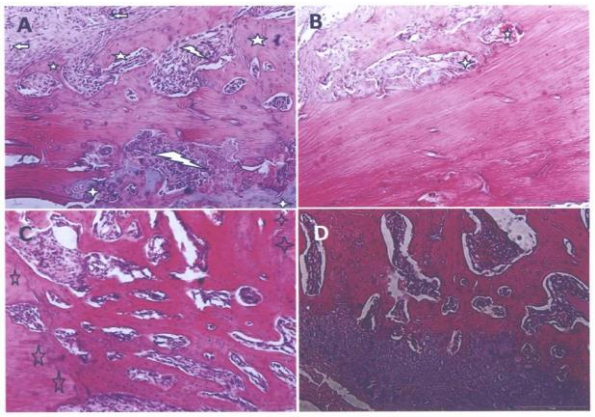

Figure 1. Donly (H and E, 40×). (a) Graft cortex incorporation as extensive resorption lacunae (lightning) with widely-spaced trabeculae of woven bone (*). Endochondral cartilaginous formation (+) and angiogenesis (arrow) is seen at the periphery. (b) Resorption starting at the periosteal surface with new woven bone. Note angiogenesis (*), osteoclasts, and osteoblasts (+) lining the surface of the lacunae. (c) Interface between host (+) and graft (*) being filled by woven bone trabeculae surrounding the resorption lacunae. (d) Endochondral callus formation with overlapping periosteal bone formation.

However, this pattern of ingrowth was more frequent in DLPST grafts. DST at the interface was inferior both at healing and/or bone formation as evident in the illustration (Figure 2f).

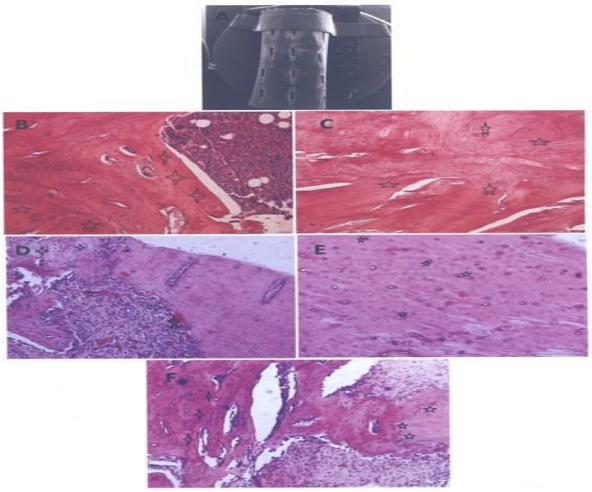

Figure 2. (a) Scanning Electron Microscopic image of laser-perforated partially demineralized allograft. (b) Demineralized laser-perforated (DLP) interface between graft (*) and host (+) filled by woven bone with narrow-spaced trabeculae and scant lacunae. (c) In DLP, laser pore completely healed by woven bone with sharp demarcations (arrows). (d) Demineralized stem cell seeded (DST) bone resorption with angiogenesis (*) in lacunae. Resorption areas lined by osteoclasts (arrow) and woven bone lined by osteoblasts and scant cartilaginous tissue (+). (e) DST, small vascular ingrowth-like porosities (*) on intact graft surfaces, a delayed separate resorption initiation. (f) DST interface with lesser healing because only a part of the new bone is in contact with graft (*) and host (+) due to higher graft resorption near junction (H and E, 40×)

In the DLPST group, a large part of the allograft was intact except traces of resorption and woven bone formation. In these grafts, degrees of biodegradation in the form of collagen fragmentation and excavation, highest number of vascular ingrowth as multiple pores throughout graft surfaces, and direct endochondral ossification of degrading grafts were seen (Figure 3a). In the DLPST grafts, a distinct bone formation at the interfaces was noted; starting with chondroblasts, subsequent sequential differentiation into classic endochondral ossification, and finally, cartilage being calcified and replaced by lamellar bone, which was seldom seen in the other three groups (Figure 3b). Callus formation in this group had either lamellar bone and/or transitional endochondral osteogenesis (Figure 3c). Healing in this group in the contact area with the graft was almost negligible or nil in most parts, and did not reach the graft bone except in two cases (Figure 3d). The highest rate of angiogenesis was evident in the vicinity of the interface areas (Figure 3c).

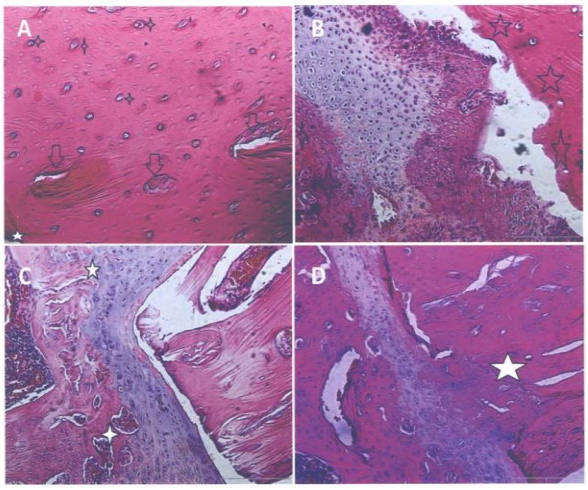

Figure 3. DLPST (H and E, 40×). (a) Most of the graft is intact. Delayed resorption initiation as porosities (+) with graft biodegradation are seen (↓). (b) Interface between graft (*) and host (+), almost replaced by endochondral bone undergoing classic endochondral sequential changes. (c) Callus (*) composed of either lamellar or endochondral bone and higher angiogenesis (+) near junction from both sides. (d) Partial healing at interface as contact area with sharp demarcation (*) with simultaneous resorption and replacement by calcified endochondral bone

Capillary angiogenesis on graft surfaces in resorption lacunae and mesenchyme connective tissue in interfaces was observed in all groups. One-way ANOVA was used to test the differences among the four groups.

Graft incorporation statistics: In graft incorporation (Table 1), the formation of new woven bone differed significantly across the four groups ($p < 0.001$). Tamhane's T2 *post hoc* comparisons of the four groups indicated that DLP grafts had a significantly higher percentage of woven bone formation than the Donly and DST groups ($p < 0.001$). The *post hoc* comparison was not possible for DLPST since no woven bone formation was observed. Comparisons were not statistically significant between Donly and DST at $p = 0.050$. Grafts that formed resorption lacunae also differed significantly ($p = 0.037$). This difference was statistically insignificant among Donly, DST, and DLP. However, DLPST had a significantly lower percent of graft resorption than Donly and DST. There were no statistically significant differences among the four groups ($p = 0.760$, 0.267) with the incorporation of grafts by cartilage and angiogenesis in resorption lacunae through capillary formation. With regard to vessel-ingrowth-like porosities at the intact graft surface, stem cell augmented laser-perforated allografts had the highest number per field area of vessel budding than any other groups, followed by demineralized stem cell-enriched grafts.

Interface statistics: The formation of interface (Table 2) woven bone between groups was statistically significant ($p < 0.001$). Both Donly and DLP grafts had a significantly greater percentage of woven bone compared to stem cell augmented groups. In contrast, comparisons within both stem cell augmented groups alike within Donly and DLP groups were non-significant. The cartilaginous tissue formation was statistically not comparable by one-way ANOVA because fewer than two groups (i.e., Donly and DLP) had shown tissue formation that was also statistically of no value. Groups also differed significantly ($p = 0.007$) in regard to angiogenesis at the interface with respect to capillaries. DLPST had more significant angiogenesis compared to Donly and DST, but there was no comparative significance between the Donly and DST groups themselves.

Table 1. Graft incorporation for each graft at 12 weeks, denoted as percent area

Feature	Donly	DST	DLP	DLPST
NWB (%)	18.59 ± 12.11	21.16 ± 14.43	53.71 ± 15.30	0
GR (%)	56.56 ± 19.76	57.94 ± 31.65	31.67 ± 19.29	93.27 ± 8.43
RL (%)	26.89 ± 11.82	26.48 ± 16.89	21.94 ± 7.55	1.10 ± 0.76
CT (%)	8.98 ± 6.46	5.51 ± 1.02	5.96 ± 3.92	7.25 ± 4.24
Number of VESperF	4.83 ± 3.50	14.87 ± 5.75	5.18 ± 2.40	34.57 ± 10.96

NWB: New woven bone; GR: Graft remnants; RL: Resorption lacunae; CT: Cartilaginous tissue; Number of VESperF: Number of vessel ingrowth like porosities per field area; SD: Standard deviation; DST: Demineralized stem cell seeded; DLPST: Demineralized laser-perforated and stem cell seeded; DLP: Demineralized laser-perforated. Variables are represented as mean ± SD

Table 2. Interface at 12 weeks postoperatively showing percent area occupied by type of tissue

Feature	Donly	DST	DLP	DLPST
iNWB (%)	53.94 ± 13.52	29.73 ± 14.59	49.99 ± 18.17	27.67 ± 11.36
iCT (%)	0	9.79 ± 5.10	8.26 ± 5.86	0
iAngio (%)	3.99 ± 1.97	1.72 ± 1.09	0	7.96 ± 4.45
ichondrCart (%)	6.27 ± 17.39	0	0	64.46 ± 21.35
Calcified Chondr (%)	13.53 ± 22.43	0	0	24.49 ± 21.54

iNWB: New woven bone formed at interface; iCT: Cartilaginous tissue at interface; iAngio: Angiogenesis at interface represented by capillary vessels filled by red blood cells; ichondrCart: Endochondral ossification tissue at stages before being calcified; Calcified Chondr: Endochondral ossification tissue at calcified stage; SD: Standard deviation; DST: Demineralized stem cell seeded; DLPST: Demineralized laser-perforated and stem cell seeded; DLP: Demineralized laser-perforated. Variables are mean ± SD

DLPST at the interface had a significantly highest percent of chondral cartilage at different stages of sequential changes. The other groups had either none at all or negligible amounts, if any, of endochondral cartilage formed at the interface.

Discussion

A large number of studies have shown the efficacy of the MSC-DBM combination as a promising composite for bone reconstitution. However, despite the encouraging results obtained, osteoinductive change shown by DBM either alone or with stem cell planting is yet to be answered (8,20). It has been shown that DBM with marrow progenitors or stem cells acts as a pseudo-periosteum and provides sufficient essential elements to initiate and accelerate bone reconstruction in fractures or bone defects (21). This was in accordance with our experiment and other studies that simple graft composites of MSC-DBM might be helpful in the reconstruction of bone defects, but this simple combination is not optimal at load-bearing sites as Cuomo et al. revealed that using MSC-DBM in critical load-bearing defects of femoral rats, these composites are not reliable for bone healing; however, they correlated it to lower concentration of MSCs and diminished or absent osteoinductive signals based on growth factors or other osteogenic stipulations (5). This experiment was also an extension of the hypothesis that the size and shape of DBM not only affects osteoinductivity but also needs to optimize its size for large defects, because small particles or powder may migrate or flow particularly in weight-bearing defects (21). This study attempted to test the effect of partially demineralized and partially DLP structural allografts, one of the potential ways to enhance graft incorporation and union at load-bearing sites.

Results showed that partially DLP grafts had substantially higher incorporation by woven bone than partially demineralized grafts; yet this difference at the interface gap remained insignificant. This result in accordance with Lewandrowski et al. demonstrated that although DLP grafts had significantly higher bone formation, they had no overall effect in terms of healing at the junction (17).

We noted that stem cell augmentation in partially DST grafts had no overall incremental effect on graft incorporation and interface when compared to partially demineralized grafts; however, the results of this group are in contrast with Shih et al., which indicated that local application of MSC to bone defect enhanced bone healing in the gap and improved bone ingrowth into the porous-coated areas in non-weight-bearing models (21). Interestingly, we also observed delayed resorption or vessel ingrowth signs in DST grafts in addition to large resorption areas, but there were no signs of either accelerated bone formation or extra osteochondral osteogenesis, which can be attributed to extra surface porosities given by laser perforations.

In DLPST grafts, we observed almost intact graft according to degrees of biodegradation in the form of collagen fragmentation and excavation and replaced endochondral bone ossification, and the highest angiogenesis, which was scarce in other groups; however, delayed resorption and loss of woven bone formation were the defects of this group. There are some evidence that describe how endochondral ossification occurs in our grafts. Based on Carnes et al. (20) Wang and Glimcher (22), when stem cells are placed in contact with DBM *in vivo*, particularly in long bone segmental defects, they usually undergo endochondral ossification and cannot differentiate into an osteoblastic lineage due to unavailability or lower sensitivity to the bioactive ingredients of DBM. It has been noted that after a certain period of time, the DBM response to unmanipulated MSC proliferation blunts and switches differentiation to osteochondrogenesis, which might affect cell survival and ultimately affect the bone growth rate due to a lack of synergistic factors (23). Furthermore, it is reported that adding growth factors such as BMP-2 and OP-1 (and other osteogenic factors such as Vitamin D3 and dexamethasone) to DBM not only has a synergistic effect on proliferation and differentiation but also optimizes osteoinduction (23,24).

Nonetheless, we believe that stem cells acted independently to increased angiogenesis and started delayed resorption extra to resorption lacunae as evident in DST. We have also observed that despite hampered osteoinductivity for a certain period, osteoconductivity can be improved by maintenance of mechanics. In conclusion, mere stem cell and

demineralized bone graft composites did not yield promising results. Surface changes induced by laser perforation accelerated angiogenesis induction by MSCs, which resulted in endochondral bone formation at the interface. Despite non-optimal results, stem cells showed a tendency to improve osteochondrogenesis, and if they could have been supplemented with the proper stipulations, the process might have improved.

Conflict of Interests

Authors have no conflict of interests.

Acknowledgments

Appreciation goes to the Department of Surgery, School of Medicine, Qom University of Medical Sciences, Qom, Iran, for providing our deeded data.

References

1. Hoffman MD, Xie C, Zhang X, Benoit DS. The effect of mesenchymal stem cells delivered via hydrogel-based tissue engineered periosteum on bone allograft healing. Biomaterials 2013; 34(35): 8887-98.
2. Ismail HD. Role of allogenic mesenchymal stem cells in the reconstruction of bone defect in rabbits. Med J Indones 2014; 23(1): 9-14.
3. Liu G, Sun J, Li Y, Zhou H, Cui L, Liu W, et al. Evaluation of partially demineralized osteoporotic cancellous bone matrix combined with human bone marrow stromal cells for tissue engineering: an in vitro and in vivo study. Calcif Tissue Int 2008; 83(3): 176-85.
4. Xie C, Reynolds D, Awad H, Rubery PT, Pelled G, Gazit D, et al. Structural bone allograft combined with genetically engineered mesenchymal stem cells as a novel platform for bone tissue engineering. Tissue Eng 2007; 13(3): 435-45.
5. Cuomo AV, Virk M, Petrigliano F, Morgan EF, Lieberman JR. Mesenchymal stem cell concentration and bone repair: potential pitfalls from bench to bedside. J Bone Joint Surg Am 2009; 91(5): 1073-83.
6. Eniwumide JO, Yuan H, Cartmell SH, Meijer GJ, de Bruijn JD. Ectopic bone formation in bone marrow stem cell seeded calcium phosphate scaffolds as compared to autograft and (cell seeded) allograft. Eur Cell Mater 2007; 14: 30-8.
7. Long T, Zhu Z, Awad HA, Schwarz EM, Hilton MJ, Dong Y. The effect of mesenchymal stem cell sheets on structural allograft healing of critical sized femoral defects in mice. Biomaterials 2014; 35(9): 2752-9.
8. Newman JT, Smith WR, Ziran BH, Hasenboehler EA, Stahel PF, Morgan SJ. Efficacy of composite allograft and demineralized bone matrix graft in treating tibial plateau fractures with bone loss. Orthopedics 2008; 31(7): 649.
9. Zimmermann G, Moghaddam A. Allograft bone matrix versus synthetic bone graft substitutes. Injury 2011; 42(Suppl 2): S16-S21.
10. Delloye C, Simon P, Nyssen-Behets C, Banse X, Bresler F, Schmitt D. Perforations of cortical bone allografts improve their incorporation. Clin Orthop Relat Res 2002; (396): 240-7.
11. Wheeler DL, Enneking WF. Allograft bone decreases in strength in vivo over time. Clin Orthop Relat Res 2005; (435): 36-42.
12. Filho CH, Kerkis I, Kerkis A, Tatsui NH, da Costa NA, Bueno DF, et al. Allogenous bone grafts improved by bone marrow stem cells and platelet growth factors: clinical case reports. Artif Organs 2007; 31(4): 268-73.
13. Nather A, David V, Teng JW, Lee CW, Pereira BP. Effect of autologous mesenchymal stem cells on biological healing of allografts in critical-sized tibial defects simulated in adult rabbits. Ann Acad Med Singapore 2010; 39(8): 599-606.
14. Ai J, Ebrahimi S, Khoshzaban A, Jafarzadeh Kashi TS, Mehrabani D. Tissue engineering using human mineralized bone xenograft and bone marrow mesenchymal stem cells allograft in healing of tibial fracture of experimental rabbit model. Iran Red Crescent Med J 2012; 14(2): 96-103.
15. Goodman SB. Allograft alternatives: bone substitutes and beyond. Orthopedics 2010; 33(9): 661.
16. Ouyang HW, Cao T, Zou XH, Heng BC, Wang LL, Song XH, et al. Mesenchymal stem cell sheets revitalize nonviable dense grafts: implications for repair of large-bone and tendon defects. Transplantation 2006; 82(2): 170-4.
17. Lewandrowski KU, Tomford WW, Schomacker KT, Deutsch TF, Mankin HJ. Improved osteoinduction of cortical bone allografts: a study of the effects of laser perforation and partial demineralization. J Orthop Res 1997; 15(5): 748-56.
18. Khojasteh A, Eslaminejad MB, Nazarian H, Morad G, Dashti SG, Behnia H, et al. Vertical bone augmentation with simultaneous implant placement using particulate mineralized bone and mesenchymal stem cells: a preliminary study in rabbit. J Oral Implantol 2013; 39(1): 3-13.
19. Park JW, Bae SR, Suh JY, Lee DH, Kim SH, Kim H, et al. Evaluation of bone healing with eggshell-derived bone graft substitutes in rat calvaria: A pilot study. J Biomed Mater Res 2008; 87A(1): 203-14.
20. Carnes DL, Jr., De La Fontaine J, Cochran DL, Mellonig JT, Keogh B, Harris SE, et al. Evaluation of 2 novel approaches for assessing the ability of demineralized freeze-dried bone allograft to induce new bone formation. J Periodontol 1999; 70(4): 353-63.
21. Shih HN, Shih LY, Sung TH, Chang YC. Restoration of bone defect and enhancement of bone ingrowth using partially demineralized bone matrix and marrow stromal cells. J Orthop Res 2005; 23(6): 1293-9.
22. Wang J, Glimcher MJ. Characterization of matrix-induced osteogenesis in rat calvarial bone defects: I. Differences in the cellular response to demineralized bone matrix implanted in calvarial defects and in subcutaneous sites. Calcif Tissue Int 1999; 65(2): 156-65.
23. Tsiridis E, Ali Z, Bhalla A, Heliotis M, Gurav N, Deb S, et al. In vitro and in vivo optimization of impaction allografting by demineralization and addition of rh-OP-1. J Orthop Res 2007; 25(11): 1425-37.
24. Klein-Nulend J, Louwerse RT, Heyligers IC, Wuisman PI, Semeins CM, Goei SW, et al. Osteogenic protein (OP-1, BMP-7) stimulates cartilage differentiation of human and goat perichondrium tissue in vitro. J Biomed Mater Res 1998; 40(4): 614-20.