Polyurethane/Hydroxyapatite Induces MSCs towards Osteo-like Cells in a Similar Fashion to Demineralized Bone Matrix

Mostafa Shahrezaei, Mohamad Moosaee[*]

Department of Orthopedic Surgery, AJA University of Medical Sciences, Tehran, Iran

Abstract

Osteoarthritis (OA) is the single most prevalent disorder in older adults having a predicted value of 130 million patients in 2050. Several clinical chemotherapeutic approaches are being applied to treat early or late osteoarthritis. It has been recommended that autologous mesenchymal stem cells (MSCs) from OA patients could be the gold standard to treat OA as these cells have high proliferation and chondrocyte lineage differentiation potential. In this work, human MSCs, derived from adipose tissue (Ad-MSCs), loaded on Polyurethane/Hydroxyapatite (PUHA) and Demineralized Bone Matrix (DBM) and their proliferation, differentiation capabilities were determined by MTT assay and Alizarin Red S staining and the expression of mRNA into osteoblast lineage were determined using Real Time PCR . The result showed that MSCs were more viable on PUHA when compared with DBM and the expression of lineage specific markers showed that differentiation potential of PUHA and DBM was not much different. The osteoblast lineage cells were stained positively with Alizarin Red S in completely similar in both groups. Electron microscopy analysis indicated attachment of Ad-MSCs when cultured on the PUHA and DBM. It was concluded that PUHA can be used in clinics as Osteo-inductive scaffold to treat OA easily, however further investigations are required before moving to clinical studies.

Keywords: Demineralized Bone Matrix, Osteoarthritis, Osteo-like Cells, Polyurethane/Hydroxyapatite

Introduction

Osteoarthritis (OA) is a long-term chronic degenerative disorder with undiscovered origin that identified by the atrophy of cartilage in joints. The main symptoms of OA are rigidity, pain and restricted movements when bones rubbed together. OA has been known as a most common disease with a worldwide distribution (Baharvand et al., 2004). According to the United Nations, people aged over 60 will account for more than 20 percent of the world's population by 2050 which are the prime victims of OA rising a danger of 130 million OA patients of whom 40 million will be disordered by the severity of disease (Bahrami et al., 2011). There are several therapeutic approaches including physical activity, surgery, medication etc. but recently stem cells especially mesenchymal stem cells (MSCs) have emerged as promising agents to treat OA because of their excessive proliferation and multilineage differentiation capability (Brodie and Humes, 2005, Caplon, 2005). Their clinical trials and promising results with enhanced healing and improved joints recovery have resulted in the applications of these cells in comparison with other cells to treat various osteo-diseases(Chakkalakal et al., 2001, Dexheimer et al., 2011). These cells can be isolated from approximately all parts of body including skin, blood, pancreas, adipose, liver, brain, lungs, heart, umbilical cord blood and kidneys. Among these issues, adipose tissue is the most common source of these kinds of stem cells with limited oncogenicity, which could be derived from the lipoasparate in routine liposuction (Friedenstein et al., 1987, García-Álvarez et al., 2011, Ghannam et al., 2010).

In comparison to the misconception that OA is easily a "wear and tear" disease, it is now obvious that pro-inflammatory cytokines and moderators are involved in the onset and advancement of this disease (Hare et al., 2009). In this regard, the potential effect of inflammatory cytokines exist in the joint space must be thought when estimating stem cell-based therapies and a good scaffold can induce precise differentiation for bone healing and recovering the damages (Hofmann et al., 2008). Scaffold based engineering may result in the generation of tissues resistant to inflammations, a major challenge in tissue engineering. Considering the importance of a scaffold, demineralized bone

*Corresponding author E-mail: *Drmoosaee@gmail.com*

graft (DBG) that is derived from the bone tissue, have been applied by researchers for bone healing and treatment of OA (Hosea Blewett, 2008).. Mineralized bone matrices with osteoconductive and osteoinductive properties (Hsieh et al., 2015, Jiang et al., 2002), have been utilized widely for the most of bone disorders with non-union characteristics (Labusca et al., 2012, Lee et al., 2008, Levenberg et al., 2003). Demineralized bone matrix is constituted of native compositions of ECM, which enhance its osteoinductive characteristics. Osteoconductive features of DBM are consequence of its collagenous network (Maetzel et al., 2004). Efficient alternatives to DBM remained a challenging step in stem cell-based treatment of osteo-diseases and it has been considered that natural derivatives could be the ideal agents for cellular seeding to induce towards osteo-like cells (Maniatopoulos et al., 1988). In this work, PUHA as a combination of PU (Polyurethane) and HA (Hydroxyapatite) has been used to seed the cells and to study cellular behavior on it. Cellular behavior has been studied and compared with DBM as a current gold standard in tissue engineering and transcriptional based approaches have been applied to find out the molecular switching (*RUNX2, COL1A1, BGLAP* and *SPARC*) of seeded MSCs on PUHA and DBM.

Materials and Methods

Isolation and characterization of Adipose MSCs

Human adipose-derived mesenchymal stem cells (Ad-MSCs) were extracted from adipose tissue as previously mentioned (Martin et al., 1999, Mulliken et al., 1981, Murphy et al., 2002, Polo-Corrales et al., 2014). Adipose tissues were isolated from three patients using liposuction surgery with written agreement at Razavi Hospital (Mashhad, Iran). Cells were separated using collagenase type I (Gibco, Australia) and MSCs were subsequently derived by centrifugation and cultured in Dulbecco's Modified Eagle's Medium-Low Glucose (DMEM-LG; Gibco, Australia) supplementary with 10% fetal bovine serum (FBS; Gibco, Australia), 100 µg/ml streptomycin and 100 U/ml penicillin (Biowest, UK). The recognition of extracted human Ad-MSCs was affirmed by their expression of particular cell surface antigens as explained before (Martin et al., 1999).

Culturing of MSCs on Scaffold

Ad-MSCs in passage three were applied for culturing on the scaffolds. Cells were separated from culture plates with trypsin/EDTA (Gibco, Australia) then added into each well. A seeding density of 1×10^6 cells was applied for both PUHA and DBM (TRC, Iran) scaffolds and 2D culture of Ad-MSCs was used as control group. In order to loading the cells in scaffolds, Ad-MSCs were resuspended in FBS. Then, all samples were incubated at 37ºC under 5% of CO_2 conditions for 15 min, after which, 1 ml of DMEM-LG supplemented with 10% FBS was added to each well. After 48h incubation, the matrices were cultured in induction media (DMEM-LG supplemented with 10% FBS, 100 µg/ml streptomycin and 100 U/mL penicillin plus 0.1 µM dexamethasone,10 mM β-glycerophosphate, and 10 mM ascorbic acid) for 21 days.

Cell Viability Assay

Cell viability assay was applied to estimate the rate of Ad-MSCs proliferation and viability. Cell-seeded on DBM and PUHA scaffolds in 12-well tissue culture plates were cultured in osteogenic induction medium for 14 and 21 days. The mitochondrial activity of ADSCs into the DBM and PUHA scaffolds can be determined by the conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (Sigma, USA) to formazan crystals. Cells were incubated in the existence of 0.5 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) for 3 hours. The optical densities (OD) of the formazan were read with a spectrophotometer at a wavelength of 570 nm. This absorbance was caused by Ad-MSCs seeded in DBM and PUHA for 14 and 21 days after culture.

Alizarin Red staining

Scaffolds were fixed with 4% paraformaldehyde, incubated with Alizarin Red S solution for 20 min. The samples were washed with distilled water and in order to quantitative analysis the scaffolds were detained in 10% acetic acid. The concentration of dye in solution was measured with the spectrophotometer at 405 nm.

Gene Expression Analysis

Total RNAs were isolated from all three groups of MSCs cultured on DBM, PUHA and control group after 21 days and cDNAs were synthesized and subjected to reverse transcription polymerase chain reaction (RT-PCR) to observe the expression of *RUNX2, BGLAP, SPARC* and *COL1A1* using specific primers as shown in Table 1. The relative expressions of osteo-lineage markers were determined using real time quantitative RT-PCR. For this purpose, synthesized cDNAs were mixed with 2x SYBR® Green Real Time PCR Master Mix (Parstous, Iran) in 8-strip tubes (Gunster Biotech,

12

Table 1. List of primers used for mRNA expression analysis using real time quantitative RT-PCR. *SPARC*: secreted protein acidic and rich in cysteine or osteonectin, *COL1A1*: Collagen, Type I, Alpha 1, *BGLAP*: Bone gamma-carboxyglutamate (Gla) protein or osteocalcin, *RUNX2*: Runt-related transcription factor 2, *TBP*: TATA binding protein gene.

Name of Gene	Forward Primer	Reverse Primer	Length of Primer
SPARC	CTGTATTCTGGTGTTCTCTTCTAC	AATGACTGAATGAGCCAATGC	170 bp
COL1A1	GACGAAGACATCCCACCAAT	CGTCATCGCACAACACCTT	124 bp
BGLAP	CTCACACTCCTCGCCCTATTG	CTCTTCACTACCTCGCTGCC	134 bp
RUNX2	CTCACTGCCTCTCACTTG	ATGTATTAACCTGGATTCTGG	163 bp
TBP	TTGTGAGAAGATGGATGTTG	AGATAGCAGCACGGTATG	151 bp

Taiwan). and the mixture was subject to CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) using following parameters: initial denaturation at 94°C for 10 minutes, 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds, followed by a final extension at 72°C for 2 minutes.

Electron Microscopy

One sample from each category was set with 2.5% glutaraldehyde solution (Sigma) for 4 hours at room temperature in order to scanning electron microscopic (SEM) analyses. After dehydration in a gradient series of ethanol (50%, 70%, 90%, 100%, for 10 min in each one), specimens were dried, coated with gold and analyzed by SEM (LEO, Germany).

Statistical Analysis

Statistical analyses were carried out by SPSS and applying one-way ANOVA test. Results are exhibited as mean values ± standard deviation. A significance value of $p < 0.05$ was approved as statistically significant.

Results

Characterization of Ad-MSCs

Flow cytometry analysis was carried out to characterize Ad-MSCs. We used 6 different antibodies against CD90, CD105, CD44, CD45, CD34 and CD11b. Majority of these cells express CD90, CD105 and CD44 markers, whereas CD45, CD34 and CD11b are expressed in low percentage of the cells (data not shown).

Cell viability

MTT assay was used in order to evaluate the rate of Ad-MSCs proliferation and viability. The cells seeded on DBM and PUHA scaffolds in osteogenic induction medium for 14 and 21 days. The OD of the formazan product produced by Ad-MSCs seeded in DBM and PUHA 14 days after culture were similar. On day 21, the viability of the cells in two types of scaffolds and control groups was similar although in PUHA group was increased, slightly (Figure 1).

Alizarin Red staining

In order to analyze calcium crystals in cells or matrix fibers, alizarin red staining carried out. The Ad-MSCs seeded on DBM and PUHA scaffolds for 21 days with osteogenic induction medium. The Alizarin Red staining of the cells culture in the two types of scaffolds was the same, as analyzed by absorbance of the solvent as shown in Figure 2.

Expression of Osteogenic genes in Ad-MSCs seeded on scaffold

At day 21, in order to examine the expression of osteogenic-related genes in the Ad-MSCs seeded in DBM, real time quantitative RT-PCRwas performed. The expressions of Runt-related transcription factor 2 (*RUNX2*), Collagen, Type I, Alpha 1 (*COL1A1*), Bone gamma-carboxyglutamate (Gla) protein or osteocalcin (*BGLAP*) and secreted protein acidic and rich in cysteine or osteonectin (*SPARC*) in relation to TATA binding protein (*TBP*) gene are shown in Figure 3. The expression of *RUNX2*, *SPARC* and *COL1A1* was increased in the Ad-MSCs seeded on DBM in presence of the induction medium, significantly. However, expression of *BGLAP* was similar in Ad-MSCs loaded on DBM and PUHA.

Electron microscopy analysis

Significant integration of cells to the DBM and PUHA scaffolds were also affirmed by SEM images.

13

Ad-MSCs attached and proliferated into well in the DBM and PUHA (Figure 4). In DBM and PUHA groups, Ad-MSCs were directly injected in the scaffolds, the cells would attach into the scaffold holes and its surface. In these groups, Ad-MSCs adhere on the scaffolds via pseudopodia. SEM was shown PUHA was better than demineralized bone graft.

Discussion

Stem cell-based tissue-engineering approaches are currently being investigated at high pace and are setting up a new era of learning that how to control the body's constitutional capacity to repair and renew skeletal tissues. For this purpose, MSCs are being introduced as reparative cells to the disorder site to fill the injury and to promote the body's self-repair by managing its intrinsically self-renewal capacity by its unmatched paracrine effects (Rosenthal et al., 1999).
Covering the challenge of autologous stem cell treatment demand, MSCs are being taken from young, healthy donors exhibiting enhanced expansion and differentiation potential for OA patients as it was hypothesized that onset of OA may decrease the chondrogenic potential of stem cells (Supronowicz et al., 2013). Studies approved that MSCs from OA patients can be derived, amplified, and differentiated into the chondrocyte lineage and then transplanted again (Thibault et al., 2009, Tran et al., 2009). However, raised age and obesity are both critical risk factors for OA and may influence the characteristic of stem cells (Hare et al., 2009, Upton et al., 1984).
Many of conventional cell culture investigations for clinical purpose have been applied in 2-dimensional (2D) culture systems; nevertheless, these culture systems are significantly limited in simulation of cell fortune in native tissues (Vilalta et al., 2008). Therefore, three-dimensional (3D) culture systems have been improved as a strategy to overcome this challenge and cells are being cultured using different scaffolds to reduce the variations in cell morphology, migration, signaling and adherence (Wang et al., 2009, Yang et al., 2015) and steps have been taken to generate a natural microenvironment *in vitro* which is more proper for investigation of cell interactions with ECM constituents (Yang et al., 2011). Biomaterial scaffolds indicated to operate as supporting structures to osteogenic cells playing a crucial role in bone tissue engineering (Yoon et al., 2007). Various types of adopted chitosan have been performed as 3D scaffolds including chitosan hydrogel and chitosan sponges to study their

potential to induce MSCs towards osteo-lineage and to keep cells viable in culturing systems (Zotarelli Filho et al., 2013).
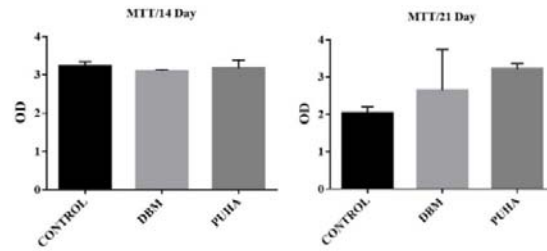
Figure 1. The proliferation and viability of adipose derived mesenchymal stem cell cultured on the PUHA and DBM (*P<0.05*).
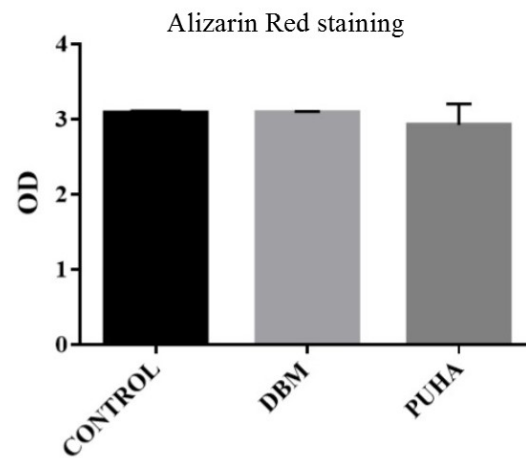
Figure 2. Alizarin Red S staining of adipose derived mesenchymal stem cell cultured on the PUHA and DBM for 14 and 21 days (*P<0.05*).
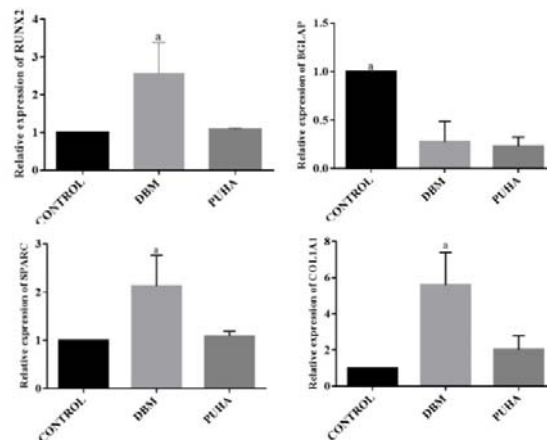
Figure 3. Gene expression of adipose derived mesenchymal stem cell cultured on PUHA and DBM (*P<0.05*).

DBM have been known as the ideal scaffold for osteo-lineage induction of stem cells but the
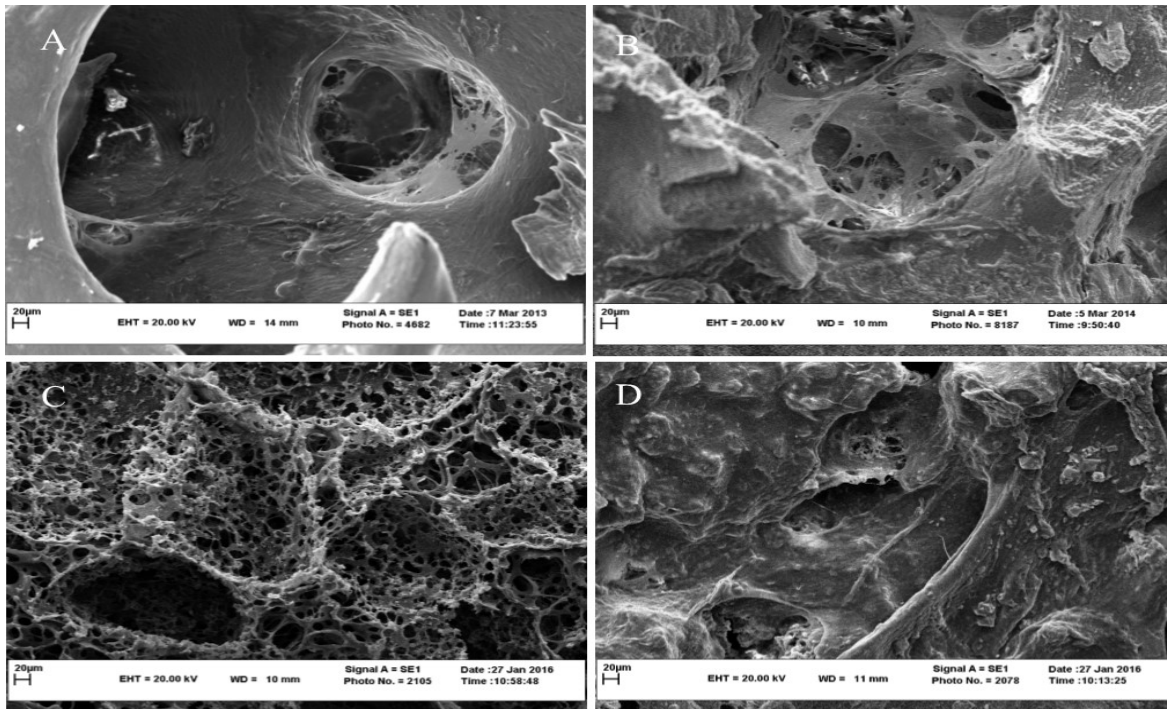
14

Figure 4. Scanning electron microscopy of PUHA and DBM. A & B) Ad-MSCs cultured on the DBM, A) in the day of seeding (500X), B) 21 days after culture in induction medium (500X), C &D) Ad-MSCs cultured on the PUHA, C) in the day of seeding (500X), D) 21 days after culture in induction medium (500X).

availability of DBM depends upon the existence of dead bone, a major challenge in the scaffold preparation (Hosea Blewett, 2008). PUHA, an ideal conjugation of PU (Polyurethane) and HA (Hydroxyl Appetite) have emerged as an ideal candidate to generate *in vitro* microenvironment as PUHA provides more vitality to cells as compared to other available scaffold matrices (Yoon et al., 2007) and an excellent three-dimensional structure for bone tissue ingrowth and cell migration and hopefully could be used as an substitute to DBM in near future for bone tissue regeneration (Zuk et al., 2002).. It has been shown that this 3D co-culture system could supply a novel foundation for the improvement of complex tissue engineered constructs with high regeneration ability (Zuk et al., 2001).. In current study, we used Ad-MSCs and studied their viability while cultured on DBM and PUHA. It was observed that PUHA is much more viable scaffold as compared to DBM for ad-MSCs whereas both scaffolds showed equal amount of osteogenic differentiation as determined by MTT and Alizan red assay respectively, as shown in Figure 1 and 2. Results obtained by qRT-PCR, confirmed the consistency of our
previously mentioned reports regarding their osteogenic differentiation (Figure 3). Considering the challenges in deriving DBM and advantages of

PUHA, it can be concluded from the current work that PUHA could be used as a novel scaffold for bone tissue engineering as there was no significant difference in cell viability, differentiation, molecular expression and electron microscopic morphology, when we compared both scaffold.

Acknowledgements

References

1. Baharvand, H., Hashemi, S.M., Ashtiani, S.K., and Farrokhi, A. (2004). Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems in vitro. Int J Dev Biol 50: 645-52.
2. Bahrami, A.R., Ebrahimi, M., Matin, M.M., Neshati, Z., Almohaddesin, M.R., Aghdami, N., and Bidkhori, H.R. (2011). Comparative analysis of chemokine receptor's expression in mesenchymal stem cells derived from human bone marrow and adipose tissue. J Mol Neurosci 44: 178-85.
3. Brodie, J.C., and Humes, H.D. (2005). Stem cell approaches for the treatment of renal

15

failure. Pharmacol Rev 57: 299-313.

4. Caplon, A. (2005). Mesenchymal stem cells: Cell based reconstructive therapy in orthopedics. Tissue Eng 11: 1198-211.

5. Chakkalakal, D.A., Strates, B.S., Garvin, K.L., Novak, J.R., Fritz, E.D., Mollner, T.J., and McGuire, M.H. (2001). Demineralized bone matrix as a biological scaffold for bone repair. Tissue Eng 7: 161-77.

6. Dexheimer, V., Mueller, S., Braatz, F., and Richter, W. (2011). Reduced reactivation from dormancy but maintained lineage choice of human mesenchymal stem cells with donor age. PloS one 6: e22980.

7. Friedenstein, A., Chailakhyan, R., and Gerasimov, U. (1987). Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. Cell Tissue Kinet 20: 263-72.

8. García-Álvarez, F., Alegre-Aguarón, E., Desportes, P., Royo-Cañas, M., Castiella, T., Larrad, L., and Martínez-Lorenzo, M.J. (2011). Chondrogenic differentiation in femoral bone marrow-derived mesenchymal cells (MSC) from elderly patients suffering osteoarthritis or femoral fracture. Arch Gerontol Geriatr 52: 239-42.

9. Ghannam, S., Bouffi, C., Djouad, F., Jorgensen, C., and Noël, D. (2010). Immunosuppression by mesenchymal stem cells: mechanisms and clinical applications. Stem Cell Res Ther 1:1.

10. Hare, J.M., Traverse, J.H., Henry, T.D., Dib, N., Strumpf, R.K., Schulman, S.P., Gerstenblith, G., DeMaria, A.N., Denktas, A.E., and Gammon, R.S. (2009). A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. J Am Coll Cardiol 54: 2277-86.

11. Hofmann, A., Ritz, U., Verrier, S., Eglin, D., Alini, M., Fuchs, S., Kirkpatrick, C.J., and Rommens, P.M. (2008). The effect of human osteoblasts on proliferation and neo-vessel formation of human umbilical vein endothelial cells in a long-term 3D co-culture on polyurethane scaffolds. Biomaterials 29: 4217-26.

12. Hosea Blewett, H.J. (2008). Exploring the mechanisms behind S-adenosylmethionine (SAMe) in the treatment of osteoarthritis. Crit Rev Food Sci Nutr 48: 458-63.

13. Hsieh, W.-C., Liau, J.-J., and Li, Y.-J. (2015). Characterization and cell culture of a grafted chitosan scaffold for tissue engineering. International Journal of Polymer Science 2015.

14. Jiang, Y., Jahagirdar, B.N., Reinhardt, R.L., Schwartz, R.E., Keene, C.D., Ortiz-Gonzalez, X.R., Reyes, M., Lenvik, T., Lund, T., and Blackstad, M. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418: 41-49.

15. Labusca, L., Zugun-Eloae, F., Shaw, G., Botez, P., Barry, F., and Mashayekhi, K. (2012). Isolation and phenotypic characterisation of stem cells from late stage osteoarthritic mesenchymal tissues. Curr Stem Cell Res Ther7: 319-28.

16. Lee, J., Cuddihy, M.J., and Kotov, N.A. (2008). Three-dimensional cell culture matrices: state of the art. Tissue Eng Part B Rev14: 61-86.

17. Levenberg, S., Huang, N.F., Lavik, E., Rogers, A.B., Itskovitz-Eldor, J., and Langer, R. (2003). Differentiation of human embryonic stem cells on three-dimensional polymer scaffolds. PANS 100: 12741-6.

18. Maetzel, A., Li, L., Pencharz, J., Tomlinson, G., and Bombardier, C. (2004). The economic burden associated with osteoarthritis, rheumatoid arthritis, and hypertension: a comparative study. Ann Rheum Dis 63: 395-401.

19. Maniatopoulos, C., Sodek, J., and Melcher, A. (1988). Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. Cell Tissue Res 254: 317-30.

20. Martin, G.J., Jr., Boden, S.D., Titus, L., and Scarborough, N.L. (1999). New formulations of demineralized bone matrix as a more effective graft alternative in experimental posterolateral lumbar spine arthrodesis. Spine 24: 637-45.

21. Mulliken, J.B., Glowacki, J., Kaban, L.B., Folkman, J., and Murray, J.E. (1981). Use of demineralized allogeneic bone implants for the correction of maxillocraniofacial deformities. Ann Surg 194: 366-72.

22. Murphy, J.M., Dixon, K., Beck, S., Fabian, D., Feldman, A., and Barry, F. (2002). Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. Arthritis Rheum 46: 704-13.

23. Polo-Corrales, L., Latorre-Esteves, M., and Ramirez-Vick, J.E. (2014). Scaffold design

for bone regeneration. J Nanosci Nanotechnol 14: 15-56.

24. Rosenthal, R.K., Folkman, J., and Glowacki, J. (1999). Demineralized bone implants for nonunion fractures, bone cysts, and fibrous lesions. Clin Orthop Relat Res 364: 61-9.

25. Supronowicz, P., Gill, E., Trujillo, A., Thula, T., Zhukauskas, R., Perry, R., and Cobb, R.R. (2013). Multipotent adult progenitor cell-loaded demineralized bone matrix for bone tissue engineering. J Tissue Eng Regen Med 10: 275-83.

26. Thibault, R.A., Scott Baggett, L., Mikos, A.G., and Kasper, F.K. (2009). Osteogenic differentiation of mesenchymal stem cells on pregenerated extracellular matrix scaffolds in the absence of osteogenic cell culture supplements. Tissue Eng Part A 16: 431-40.

27. Tran, C., Ouk, S., Clegg, N.J., Chen, Y., Watson, P.A., Arora, V., Wongvipat, J., Smith-Jones, P.M., Yoo, D., and Kwon, A. (2009). Development of a second-generation antiandrogen for treatment of advanced prostate cancer. Science 324: 787-90.

28. Upton, J., Boyajian, M., Mulliken, J.B., and Glowacki, J. (1984). The use of demineralized xenogeneic bone implants to correct phalangeal defects: a case report. J Hand Surg Am 9: 388-91.

29. Vilalta, M., Dégano, I.R., Bagó, J., Gould, D., Santos, M., García-Arranz, M., Ayats, R., Fuster, C., Chernajovsky, Y., and García-Olmo, D. (2008). Biodistribution, long-term survival, and safety of human adipose tissue-derived mesenchymal stem cells transplanted in nude mice by high sensitivity non-invasive bioluminescence imaging. Stem Cells Dev 17: 993-1004.

30. Wang, L., Li, Y., Zuo, Y., Zhang, L., Zou, Q., Cheng, L., and Jiang, H. (2009). Porous bioactive scaffold of aliphatic polyurethane and hydroxyapatite for tissue regeneration. Biomed Mater 4: 025003.

31. Yang, W., Both, S.K., Zuo, Y., Birgani, Z.T., Habibovic, P., Li, Y., Jansen, J.A., and Yang, F. (2015). Biological evaluation of porous aliphatic polyurethane/hydroxyapatite composite scaffolds for bone tissue engineering. J Biomed Mater Res A 103: 2251-59.

32. Yang, Z., Wu, Y., Li, C., Zhang, T., Zou, Y., Hui, J.H., Ge, Z., and Lee, E.H. (2011). Improved mesenchymal stem cells attachment and in vitro cartilage tissue formation on chitosan-modified poly (L-lactide-co-epsilon-caprolactone) scaffold. Tissue Eng Part A 18: 242-51.

33. Yoon, E., Dhar, S., Chun, D.E., Gharibjanian, N.A., and Evans, G.R. (2007). In vivo osteogenic potential of human adipose-derived stem cells/poly lactide-co-glycolic acid constructs for bone regeneration in a rat critical-sized calvarial defect model. Tissue Eng 13: 619-27.

34. Zotarelli Filho, I.J., Frascino, L.F., Greco, O.T., de Araújo, J.D., Bilaqui, A., Kassis, E.N., Ardito, R.V., and Bonilla-Rodriguez, G.O. (2013). Chitosan-collagen scaffolds can regulate the biological activities of adipose mesenchymal stem cells for tissue engineering. journal of Regenerative Medicine and Tissue Engineering 2: 12.

35. Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., Benhaim, P., and Hedrick, M.H. (2002). Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13: 4279-95.

36. Zuk, P.A., Zhu, M., Mizuno, H., Huang, J., Futrell, J.W., Katz, A.J., Benhaim, P., Lorenz, H.P., and Hedrick, M.H. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7: 211-28.

Open Access Statement: