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Original Article

Improved osteogenic differentiation of umbilical cord blood MSCs using custom made perfusion bioreactor

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ABSTRACT

Background: 3D cell culture is an appropriate method to develop engineered bone tissue, where different bioreactors have been designed to mitigate the challenges in 3D culture. Currently, we tailored a perfusion reactor to witness human mesenchymal stem cells (MSCs) proliferation and differentiation over polylactic acid-polyethylene glycol (PLA/PEG) composite scaffolds.
Methods: The composite scaffolds with different weight ratios of PLA and PEG were prepared using solvent casting-particulate leaching technique. Human umbilcal card blood MSCs were cultured under dynamic and static conditions to elucidate the role of dynamic fluid flow in osteogenesis of MSCs.
Results: The human MSCs distribution over the scaffolds was confirmed with fluorescent microscopy. Alkaline phosphatase (ALP), calcium mineralization, and collagen formation were found to be higher in PLA90 scaffolds than PLA100 and PLA75. PLA90 scaffolds with better cell adhesion/proliferartion were considered for bioreactor studies and they exhibited enhanced ALP, Ca^{+2} mineralization and collagen formation under dynamic perfusion than static culture. We further confirmed our observation by looking at expression levels of osteogenic marker (Runx2 and osteonectin) in differentiated MSCs subjected to perfusion culture compared to static culture.
Conclusion: The results of the current investigation once again proves that dynamic perfusion cultures improve the osteogenic differentiation of MSCs over hybrid polymer scaffolds (PLA90) for effective bone regeneration.

The development of a successfully engineered bone graft achieved by culturing stem cells on an appropriate scaffold which should support cell adhesion, proliferation, and differentiation into osteoblast cells. Selection of suitable scaffold material and stem cell source are vital factors for engineered tissue synthesis including bone tissue [1]. The ethical and social issues pertained to use the embryoinic stem cells in tissue engineering research has encouraged the application of human umbilical cord blood-derived mesenchymal stem cells

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At a glance of commentary

Scientific background on the subject

Tissue engineering is widely progressed from the field of biomaterials where combination of implants, cells, and biologically molecules gives rise to a 3D functional tissue constructs. These constructs restore and improve damaged tissues or whole organs. The success stories of artificial grafts hold the smaller skin and cartilage implants approved by the FDA; however, cures for complex, often chronic diseases is a distant goal to be achieved. One of the wide medical applications of bone tissue engineering is developing a successful bone grafts.

What this study adds to the field

To the best of our knowledge, fluid flow effect on bone regeneration using larger scaffolds in perfusion system is not yet reported. This reactor model and studies could help in optimizing the mechanical micro environment for larger scale culture of tissue engineered bone.

(MSCs) as an alternative. Along with this, the vast abundance and non-invasive procurement of MSCs from cord blood source potentially increased their use in bone tissue engineering [2,3]. The chemical environment determines the MSCs differentiation lineage, especially the growth factors as they trigger the specific signalling events involved in the pathways [4–6]. Many research studies confirmed the mechanical stimuli also play a significant role in enhancing osteogenic differentiation of MSCs [7–9].

Thus, the dynamic cultures using bioreactors have gained more attention for engineered bone tissue development. Bioreactor culture provides mechanical stimulation, and adequate nutrient supply throughout the three dimensional (3D) construct to produce large bone tissue. Ideally, human cells are subjected to physical and chemical cues in the human body constantly to ensure their functional role in tissue regeneration [10–13]. Various bioreactors such as rotating wall vessel (RWV), spinner flask, and perfusion bioreactors are developed for regenerative bone studies, the limitations of RWV and spinner flask bioreactors mitigated by using perfusion bioreactors [14–16].

Generally, perfusion bioreactors generate shear stress which enhances the osteogenic differentiation of MSCs, however, these reactors are restricted to the smaller sized scaffolds, though the huge bone defects demand the employment of bigger scaffolds. This problem underscores the importance of optimizing the culture conditions in a bioreactor for constructing larger tissues. To address this issue, we developed a customized, autoclavable, and cost-effective perfusion bioreactor which can be used with the PLA/PEG scaffolds of dimensions 30 mm diameter, 5 mm thickness. We hypothesized that the fluid flow could improve the osteogenic differentiation of MSCs. The results of the study will contribute in elucidating the significance of mechanical stimuli via fluid flow on osteogenic differentiation of MSCs on the larger scaffold.

Materials and methods

Polylactic acid (PLA) (Natureworks®, USA), Poly(ethylene glycol), Dichloromethane, DMEM-Dulbecco's Modified Eagle's medium, FBS-Fetal bovine serum (FBS), Porcine skin gelatin, Phosphate buffer saline (PBS), Penicillin, Glutaraldehyde, dexamethasone, L-Ascorbic acid and Dimethyl Sulphoxide were procured from Sigma–Aldrich.

Scaffold preparation

Composite PLA/PEG scaffolds were fabricated using solvent casting-particulate leaching technique. NaCl (300–400 μm) used as porogen. Varied weight ratios 100:0, 90:10, and 75:25 of PLA and PEG were dissolved in 30 mL of dichloromethane. Porogen mixed with polymer blended solution in 1:9 ratio. Then, poured in a petri dish and vacuum dried for 48 h for solvent evaporation. In order to leach out the salt, scaffolds were immersed in deionized water for 2–3 days by changing the water for every six hours. The prepared scaffolds were cut into definite desired dimensions using a laser cutter (Mini 19 Laser, Epilog Laser). 0.1 w/v% porcine skin gelatin coated scaffolds were sterilized by immersing them in 70% ethanol. Then, scaffolds were washed with PBS three times and incubated in culture medium for 12 h prior cell seeding. 100/0-PLA/PEG, 90/10- PLA/PEG, and 75/25-PLA/PEG are denoted as PLA100, PLA90, and PLA75 respectively.

Cell culture and osteogenic differentiation

Umbilical cord blood MSCs (5×10^4 cells/mL) were seeded onto scaffolds and incubated for 2 h to facilitate cell adhesion to the scaffold in basal media (BM) (DMEM with 10% FBS and 1% penicillin). Then, cell-seeded scaffolds were immersed in BM and incubated for 2 days. Osteogenic induction media (OIM) (BM, 50 μg/mL of Ascorbic acid, 5 mM β-glycerol phosphate, and 100 nM dexamethasone) added to the scaffolds on day 3; cells were allowed to grow/differentiate for 21 days by changing the osteogenic induction media for 2–3 days.

Osteogenic differentiation under dynamic culture conditions

PLA90 scaffolds (size: diameter × thickness 30 mm × 5 mm) used for bioreactor culture. Bioreactor used in this study shown in Fig. 1. 5×10^5 MSCs were seeded onto the scaffold and incubated for 2 h. BM was added to the scaffolds and incubated for 3 days. To investigate static and dynamic culture effect on osteogenic differentiation of MSCs, ($n = 4$) scaffolds were transferred to bioreactor chamber and ($n = 4$) scaffolds were kept in static condition. The media was supplied with a flow rate of 13.9 mL/min, resulting in a flow of 3.47 mL/min to each bioreactor. Whereas, the media was changed every 2–3 days for static scaffolds. At the end of incubation time, samples prepared for osteogenic assays and metabolic activity.

Resazurin reduction (RR) assay

Resazurin reduction (RR) was employed to check the cell viability on scaffolds. The scaffolds were immersed in RR

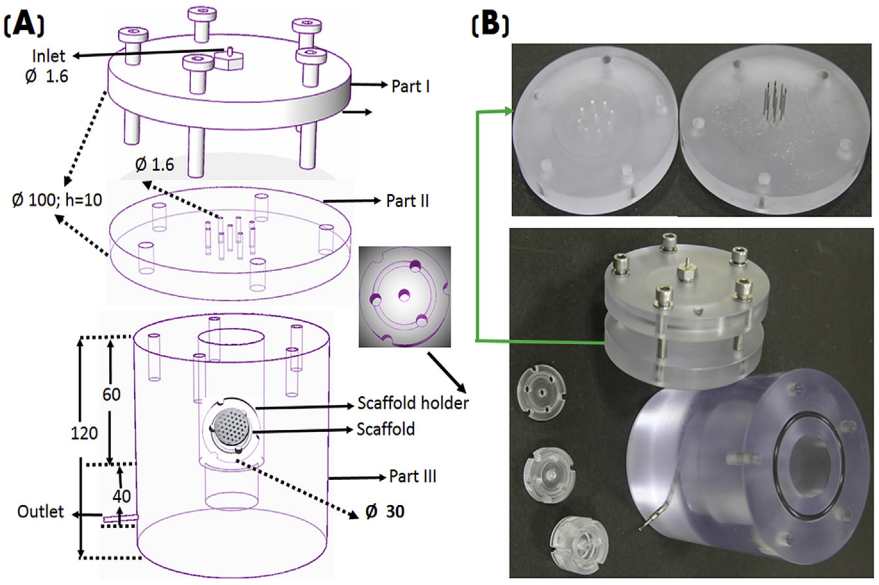

Fig. 1 Bioreactor used for dynamic culture. (A) Schematic representation of perfusion bioreactor (B) Custom made perfusion bioreactor.

working solution (10 vol % of RR stock solution in BM, stock solution is prepared by dissolving 1 mM RR sodium salt in distilled water) in the new well plate. Then, well plates were wrapped in foil, incubated for 4 h. Reduced solution of 200 μl was taken into a 96-well plate in triplicate and readings were taken using a plate reader at an excitation wavelength of 540 nm and an emission wavelength of 630 nm.

Osteogenic assays

Scaffolds were washed twice in PBS after removal of culture medium. Cell digestion buffer (10 v/v% cell assay buffer (1.5 M Tris–HCl, 1 mM $ZnCl_2$, 1 mM $MgCl_2$ in deionized water (diH_2O)), 1% TritonX100, in diH_2O) was added to each scaffold and incubated for 45 min. Scaffolds with cell lysate were vortexed, overnight stored at 4 °C. Three freeze–thaw cycles (80 °C 10 min, 37 °C 15 min) performed, then centrifuged at 10,000 rpm for 5 min. 20 μL of the lysate and 180 μL of PNPP Phosphatase Substrate added to a 96 well plate. Then, incubated at room temperature until the slight yellowish color was observed [17]. The readings recorded at a wavelength of 405 nm every minute for 30 min. This activity is normalized to the amount of DNA as determined using the DNA quantification assay.

Quant-iT™ dsDNA assay kit was used to calculate the amount of DNA (Thermo Fisher). 10 μL of the lysate added to 90 μL of working solution. The reading is taken at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. A standard curve was used to convert the fluorescence to nano-gram (ng) of DNA.

Alizarin red S was used to measure the calcium deposition by the cells. Initially, samples were washed twice with PBS. 2 mL of ARS working solution (1 w/v% ARS dissolved in diH_2O) was added to each scaffold and kept for 30 min. Then, washed with diH_2O every 5 min after removal of ARS solution until the water remained clear. The samples were submerged in 5% of perchloric acid, left for 15 min with gentle orbital shaking to destain the samples. A destained solution of 150 μL was taken to measure the absorbance readings at a wavelength of 405 nm; standard curve was used to convert absorbance values to ARS concentration (μg/mL).

DR80 assay elucidates the total collagen content (based on Sirius red). After performing ARS, samples were washed thrice with water. DR80 working solution (1 w/v% direct red 80 in saturated picric acid) of known volume was added to each sample, then left for 18 h with orbital shaking at 100 rpm. 0.2 M NaOH: CH_3OH (1:1) was added to each sample and left for 20 min over an orbital shaker at 100 rpm. The absorbance of 150 μL of destained solution was measured using plate reader at 405 nm. The DR80 concentration was (μg/mL) calculated using a standard curve.

Western blotting

Proteins from Differentiated osteoblasts were separated by SDS- polyacrylamide gels and electrotransferred to PVDF membrane. The membranes were blocked with 5% non-fat dry milk in TBS-Twin for 1 h at room temperature. The membranes were then incubated overnight at 4 °C with antisera raised against Runx2, osteonectin and beta-galactosidase in TBS-Twin containing 1% non-fat dry milk. The membranes were washed thrice for 5 min each with TBS-T and probed with appropriate near-Infrared Fluorescent Secondary Antibodies (LI-COR) in a 1% non-fat dry milk in TBS-T at room temperature for 1 h. After washing, the immune complexes comprising of the target proteins were detected using the Odyssey imaging system from LI-COR. The membranes then were stripped and reprobed with GAPDH controls. The band intensity was quantified and protein expression levels were calculated relative to GAPDH from the same membrane.

Fluorescent images

MSCs over the polymer scaffold surface were fixed using ice-cold ethanol for 10 min, and the fixative was allowed to evaporate at room temperature. These scaffolds were further washed in 1X PBS and allowed to rest in the 1X PBS buffer. ActinGreen™ 488 (ThermoFisher) reagent was added to the buffer solution (2 drops/mL) and incubated for 30 min before observing under a confocal microscope.

Statistical analysis

GraphPad Prism 7 software was employed for statistical analysis. The data represented in Mean ± SD.

Results and discussion

Cell viability on composite scaffolds

RR reduction assay elucidated the metabolic activity of the cells, and the results depicted in Fig. 2. On first day, in comparison with PLA and PLA75 scaffolds PLA90 have shown improved cell adhesion. As the time pass by, cell proliferation improved over all the scaffold types [Fig. 2B]. However, no significant variation in the cell viability was found in all the composite scaffolds on day 4. The disparity in cell attachment on the scaffold was due to the varied composition of PLA and PEG, as it was reported earlier that cell adhesion is affected by PLA content in composite scaffolds [18]. Cell adhesion could be higher on hydrophilic polymers, as PEG addition increases the hydrophilic nature resulted in cell adhesion in PLA90 scaffold [19]. However, PLA75 showed lesser cell adhesion than PLA90, which could be due to larger pores presented in the scaffold. From day 7 onwards, there was a profound increase in cell number on PLA90 scaffold, which is statistically significant ($p < 0.0001$) than other scaffolds. Cell viability was significantly higher in PEG contained scaffolds than 100% PLA scaffold. It was reported the higher cell viability on PEG/PLA electrospun fibrous scaffolds achieved, which is supported by enhanced cell attachment and also favoured for cell proliferation and penetration into the scaffold [20]. Though hMSCs adhesion rate differs concerning the composition of the scaffold, cell viability on the scaffold is not in line with the cell adhesion trend. The hMSCs were well distributed over PLA-PEG scaffolds, PLA90 showed the greater distribution compared to PLA100 and PLA75 [Fig. 3].

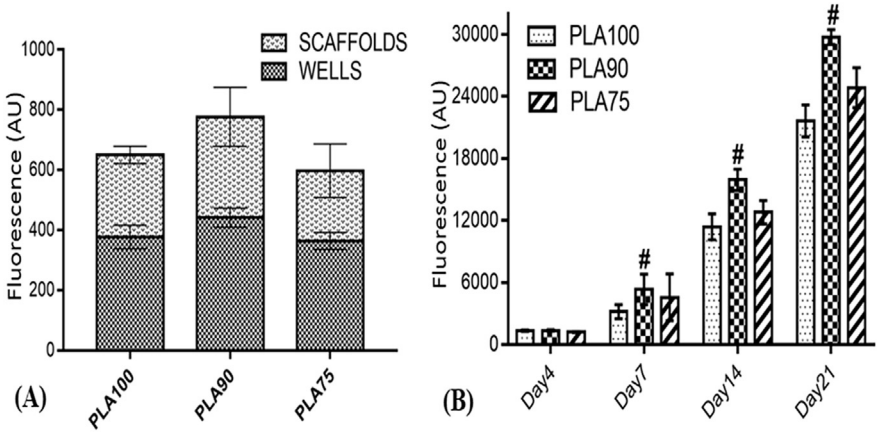

Fig. 2 Cell viability on PLA-PEG composite scaffolds. (A) Cell adhesion/distribution over scaffold and control surface on day 1 (B) Fluorescent detection of reduced RR solution from MSCs cultured over assorted saffolds at different time periods. Cell viability significantly higher on scaffold PLA90 than other scaffolds (n = 6, Mean ± SD, $p < 0.0001$).

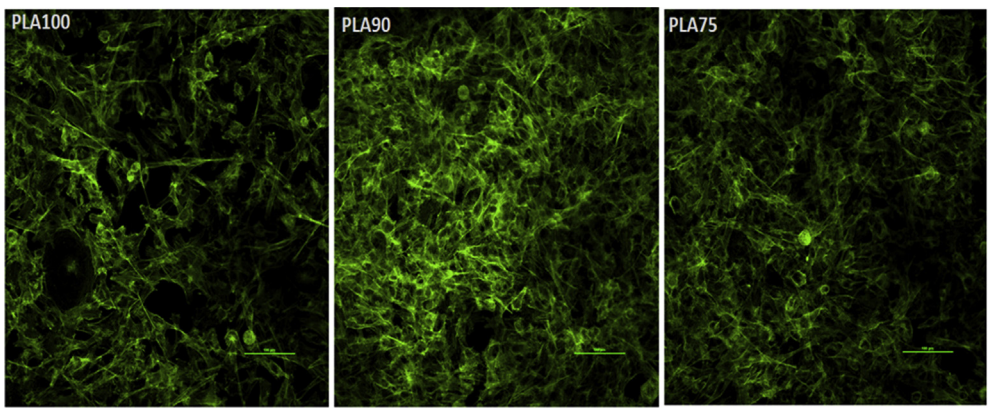

Fig. 3 Fluorescence staining of hMSCs cultured on composite scaffolds PLA100, PLA90, and PLA75 on day 14.

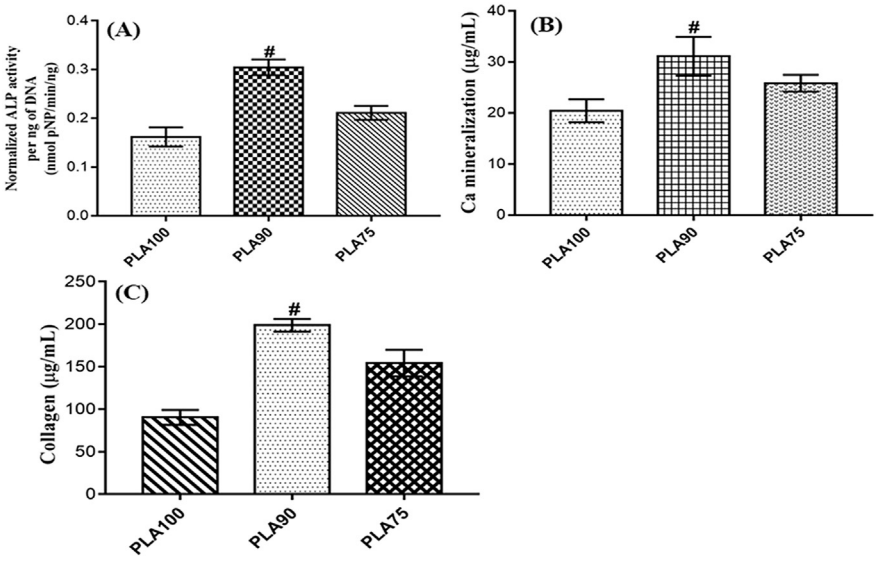

Fig. 4 Osteogenic differentiation of hMSCs. (A) Normalized ALP activity per DNA content on composite scaffolds on day 14 (n = 6). (B) Calcium deposition (μg/mL) on scaffolds measured at day 21 (C) On day 21, collagen formation on composite scaffolds. All the data represented in Mean ± SD (n = 6, $p < 0.05$). Statistical significance was represented by '#'.

Osteogenic differentiation of hMSCs on composite scaffolds

Normalized ALP activity per DNA content is shown in Fig. 4A. PLA90 scaffold exhibited higher ALP normalized activity than PLA100 and PLA75. The osteogenic response was varied on composite scaffolds; it could be due to the scaffold internal architectures [21]. A significant higher ALP activity was earlier reported in PEG-containing PLA scaffold [22], and the same trend was shown in the current study, which confirmed the early osteogenic differentiation of MSCs on composite scaffolds. Ni et al. confirmed that calcium mineralization was higher in PEG contained scaffolds, in the similar way calcium mineralization was found to be higher in PEG contained scaffolds compared to PLA only scaffold [Fig. 4B]. As like calcium mineralization, the similar trend observed in collagen formation. The results of this study confirmed that osteogenesis was higher in PLA90 scaffolds than PLA100 and PLA75. Thus, PLA90 scaffolds were chosen for further to elucidate the static and dynamic culture effect on the osteogenic differentiation of hMSCs using perfusion bioreactor.

Dynamic fluid flow subjected to MSCs

The metabolic activity of cells showed higher fluorescence values in the scaffolds with fluid flow than static culture [Fig. 5]. Fluorescent images of cells stained with "ActinGreen 488" under static and dynamic conditions depicted the better cell distribution over scaffolds cultured in dynamic culture conditions. In addition we also checked for cell survival efficiency in both static and dynamic culture conditions by quantifying the expression of beta-galactosidase (a senescence marker) and we observed a minimal beta-gal expression confirming that cell proliferation/survival in both the culture conditions was unaffected [Fig. 6]. A statistically significant ALP per DNA activity in PLA90 scaffold under dynamic culture noticed than static culture [Fig. 7A]. The ALP activity and bone matrix deposition under perfusion culture showed higher [23]. Western blotting of terminally differentiated MSCs further supported our observations seeded over PLGA scaffolds showed increased osteocalcin (OCN) and Runx2 expression [Fig. 8], and our observations confirmed that osteogenic differentiation enhanced by applied mechanical stimuli [24]. Calcium mineralization on PLA90 scaffold under dynamic condition was significantly higher than static [Fig. 7B]. MSCs cultured on starch-based scaffolds subjected fluid flow showed increased bone-like matrix deposition, which elicits mechanical stimuli significantly affect the osteogenic potential of MSCs [25]. Calcium mineralization and collagen formation were higher in reactor culture than static culture; this demonstrates mechanical stimuli directs osteogenesis [26]. On day 21, the collagen formation is also in a similar trend in line with calcium mineralization under static and dynamic culture. Collagen formation is significantly higher in dynamic culture $p < 0.0017$ than static culture. Fluid flow without osteogenic induction supplements in media showed higher

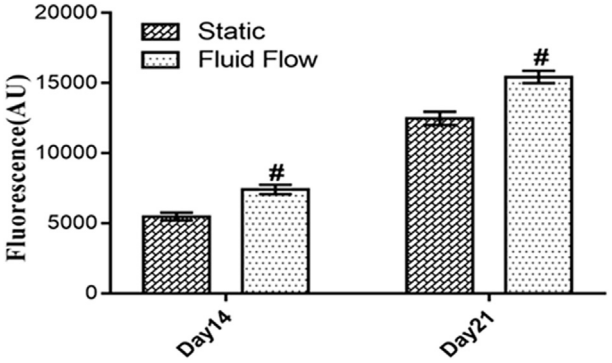

Fig. 5 Effect of static and dynamic conditions on cell viability on PLA90 scaffold at different time points. # indicates statistical significance, $p < 0.0001$, n = 3.

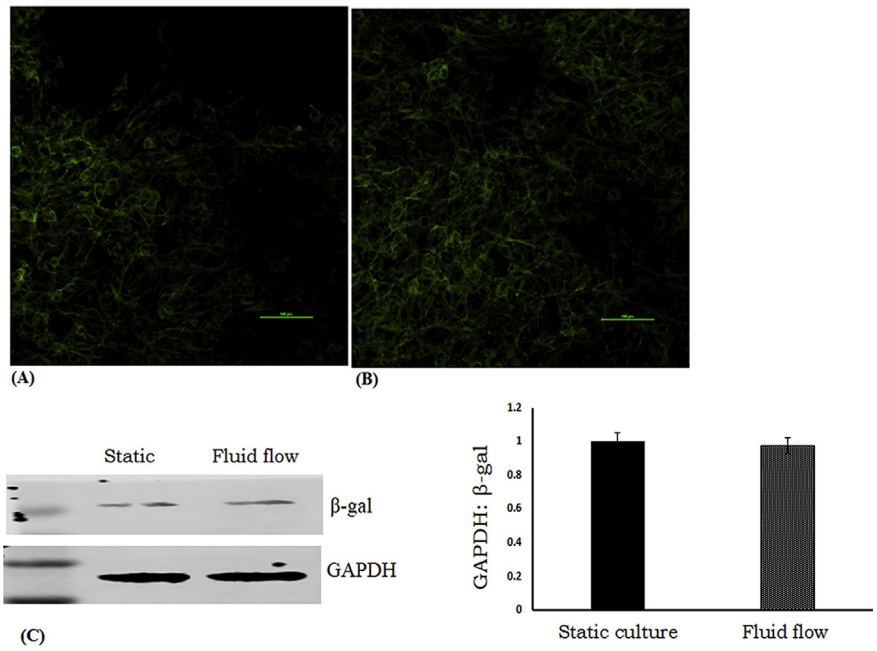

Fig. 6 Fluorescence images of cells on day 7. Cell distribution over scaffold under (A) static (B) bioreactor culture. (C) Western blots of MSC cultures, both from static and fluid flow cultures. We observed very faint β-galactosidase bands in both static and dynamic cultures showing minimal senescence in both the culture conditions.

matrix deposition by MSCs seeded on 3D macro-porous zirconium dioxide ceramic scaffold [27]. Dynamic perfusion culture of MG-63 osteoblast cells cultured on chitosan scaffold results showed an increase in bone formation, which confirms with higher ALP activity, and collagen formation [28]. The results of the current studies are also clearly confirming fluid flow enhancing osteogenesis of MSCs.

The proposed reactor model is autoclavable; thus provides a sterile environment throughout the experimental study; the flow delivery to the scaffold can be made without any bubble formation and easy to operate. Though many perfusion bioreactors have been developed to date, they are restricted to smaller size scaffolds, the major disadvantage to fill the large bone defects. We developed a perfusion bioreactor for larger

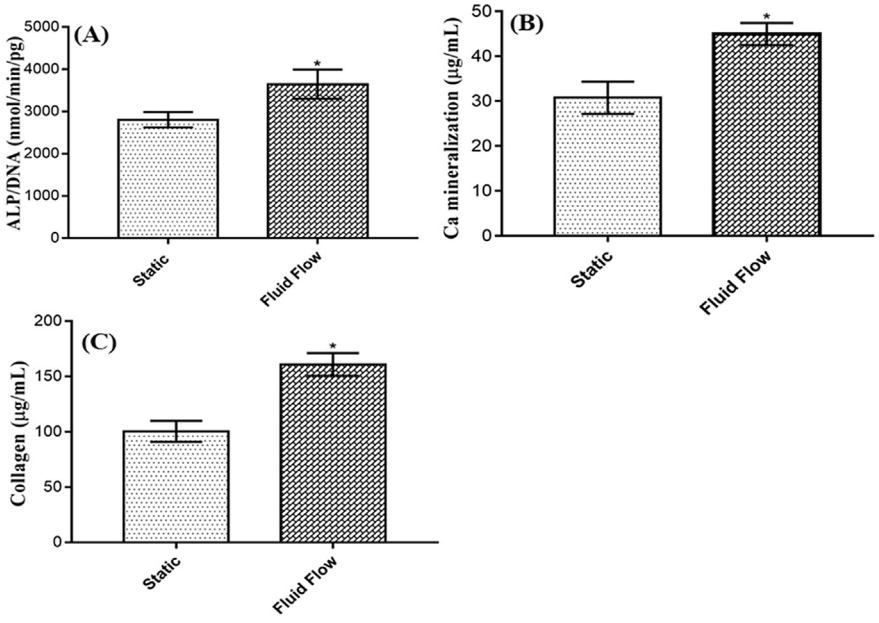

Fig. 7 Osteogenic differentiation of hMSCs under static and dynamic culture is elucidated by using osteogenic assays. (A) ALP normalized activity, for the DNA content under static and fluid flow conditions on day 14. (B) Deposition of calcium on PLA90 scaffold under static and dynamic conditions at day 21. (C) Variation in collagen formation on scaffolds under static and dynamic culture at day 21. (n = 3, Mean ± SD).

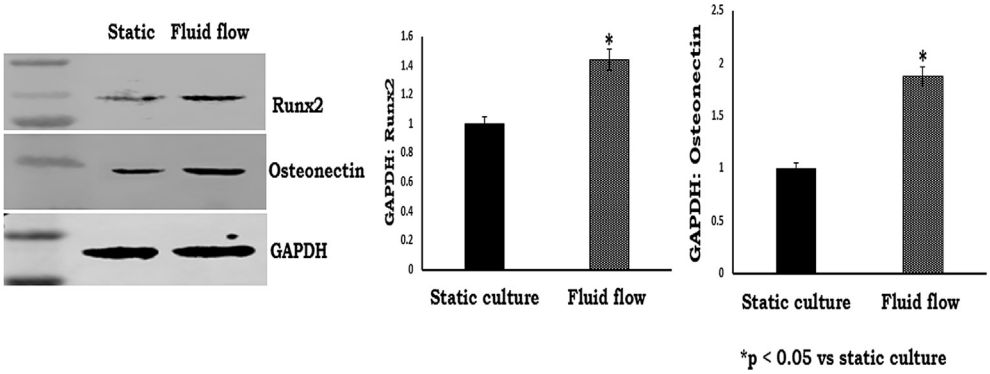

Fig. 8 Western blots of MSC cultures, both from static and fluid flow cultures. We saw a significant increase of osteogenic marker "Runx2 and osteonectin" expression in dynamic fluid flow cultures.

bone tissue engineering scaffolds; interestingly, this can be adapted to different size and shape of the scaffolds. The design aspects and significance of the model are discussed in detail in the previous work [29]. Our earlier work using this bioreactor confirmed that this bioreactor can be operated smoothly and can be used for applying FF to stimulate the bone regeneration. However, it is very important to authenticate this reactor model by using different scaffold and cell combinations to confirm whether the FF applied in this reactor can support the bone formation. Thus, the present investigations have taken up to prove the role of FF in the bone formation using the developed in-house bioreactor. Starch-based PCL scaffolds with a dimension of 8 mm diameter and 1.5–2 mm height were used to culture cells in perfusion bioreactor and concluded that FF plays a vital role in engineered bone graft development [25]. However, the perfusion bioreactor chosen in their study is not suitable for larger scaffolds, this can be addressed with our perfusion reactor which also provides uniform flow delivery to the scaffold through multi channel inlet system [25] Meinel et al. reported that dynamic medium flow improved the bone mineralization which was confirmed by higher calcium deposition over silk scaffolds (6.4 mm diameter × 1.5 mm thick discs) [30]. The up-regulation of osteogenic markers expression under perfusion regimen had been reported earlier [9]. In our study also, we found that key osteogenic markers RunX2 and osteonectin, was higher (as known from the enhanced corresponding mRNA level) under applied FF. In this study, the higher ALP activity and mineralization showed by the MSCs under dynamic culture condition, the results are in agreement with Nguyen et al., where ALP activity showed higher by human mesenchymal progenitor cells (hMPCs) [31]. In another study, MC3TC3 (pre-osteoblast) cells were cultured on chitosan-hydroxyapatite (HA) scaffolds (6 mm diameter × 4 mm thickness circular discs) using static method. The perfusion culture showed greater cell viability and osteogenic differentiation of MC3TC3 (pre-osteoblast cells) compared to static culture [32]. Though, we investigated the osteogenic potential of MSCs under perfusion culture, proved that FF could enhance the osteogenesis. Interestingly, no reports have been noted so far on larger scaffolds, we had been previously reported the static seeding combined with perfusion culture improved osteogenic differentiation of hESMPs. Our reactor model can be used for a variety of scaffold/cell combinations to elucidate the significant role of FF shear stress on bone regeneration to address the challenges in tissue formation. Also, it can be used for other tissue engineering applications.

Conclusion

After carrying out a series of experiments over PLA/PEG scaffolds under static culture conditions, greater cell viability found in PEG contained scaffold. PEG contained scaffolds showed the higher ALP, calcium, and collagen than pure PLA, and PLA90 showed the best among all scaffolds. Perfusion bioreactor culture results compared with static culture, we have found that enhanced osteogenic differentiation MSCs under dynamic conditions, which was confirmed by higher ALP, Ca^{+2}, and collagen activities. Superior Runx2 and osteonectin expression in dynamic fluid flow suggests that FF has a significant role in enhanced bone formation. The developed perfusion reactor is cost-effective, easy to operate, and autoclavable bioreactor, which will have great potential application in bone tissue engineering to understand the physiological cues role in MSCs differentiation.

Ethical statement

We declare that there are no ethical issues for human or animal rights in the work presented here.

Conflicts of interest

The authors have no financial conflicts of interest.

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REFERENCES

[1] Kedong S, Wenfang L, Yanxia Z, Hong W, Ze Y, Mayasari L, et al. Dynamic fabrication of tissue-engineered bone substitutes based on derived cancellous bone scaffold in a spinner flask bioreactor system. Appl Biochem Biotechnol 2014;174:1331–43.

[2] Lo YP, Liu YS, Rimando MG, Ho JHC, Lin K, Lee OK. Three-dimensional spherical spatial boundary conditions differentially regulate osteogenic differentiation of mesenchymal stromal cells. Sci Rep 2016;6:21253.

[3] Mekala NK, Baadhe RR, Rao PS, Devi YP. Enhanced proliferation and osteogenic differentiation of human umbilical cord blood stem cells by L-ascorbic acid, in vitro. Curr Stem Cell Res Ther 2013;8:156–62.

[4] Mu C, Lv T, Wang Z, Ma S, Ma J, Liu J, et al. Mechanical stress stimulates the osteo/odontoblastic differentiation of human stem cells from apical papilla via ERK 1/2 and JNK MAPK pathways. BioMed Res Int 2014:494378.

[5] Wu X, Hou T, Luo F, Xing J, He Q, Jin H, et al. Vascular endothelial growth factor and physiological compressive loading synergistically promote bone formation of tissue-engineered bone. Tissue Eng Part A 2013;19:2486–94.

[6] Bhaskar B, Mekala NK, Baadhe RR, Rao PS. Role of signaling pathways in mesenchymal stem cell differentiation. Curr Stem Cell Res Ther 2014;9:508–12.

[7] Chen JC, Jacobs CR. Mechanically induced osteogenic lineage commitment of stem cells. Stem Cell Res Ther 2013;4:107.

[8] Delaine-Smith RM, Reilly GC. Mesenchymal stem cell responses to mechanical stimuli. Muscles Ligaments Tendons J 2012;2:169–80.

[9] Filipowska J, Reilly GC, Osyczka AM. A single short session of media perfusion induces osteogenesis in hBMSCs cultured in porous scaffolds, dependent on cell differentiation stage. Biotechnol Bioeng 2016;113:1814–24.

[10] Bilodeau K, Mantovani D. Bioreactors for tissue engineering: focus on mechanical constraints. A comparative review. Tissue Eng 2006;12:2367–83.

[11] Kim J, Ma T. Bioreactor strategy in bone tissue engineering: pre-culture and osteogenic differentiation under two flow configurations. Tissue Eng Part A 2012;18:2354–64.

[12] Duncan RL, Turner CH. Mechanotransduction and the functional response of bone to mechanical strain. Calcif Tissue Int 1995;57:344–58.

[13] Mitra D, Whitehead J, Yasui OW, Leach JK. Bioreactor culture duration of engineered constructs influences bone formation by mesenchymal stem cells. Biomaterials 2017;146:29–39.

[14] Yeatts AB, Fisher JP. Bone tissue engineering bioreactors: dynamic culture and the influence of shear stress. Bone 2011;48:171–81.

[15] Mekala NK, Baadhe RR, Potumarthi R. Mass transfer aspects of 3D cell cultures in tissue engineering. Asia Pac J Chem Eng 2014;9:318–29.

[16] Salehi-Nik N, Amoabediny G, Pouran B, Tabesh H, Shokrgozar MA, Haghighipour N, et al. Engineering parameters in bioreactor's design: a critical aspect in tissue engineering. BioMed Res Int 2013:762132.

[17] Owen R, Sherborne C, Paterson T, Green NH, Reilly GC, Claeyssens F. Emulsion templated scaffolds with tunable mechanical properties for bone tissue engineering. J Mech Behav Biomed Mater 2016;54:159–72.

[18] Moran JM, Pazzano D, Bonassar LJ. Characterization of polylactic acid–polyglycolic acid composites for cartilage tissue engineering. Tissue Eng 2003;9:63–70.

[19] Webb K, Hlady V, Tresco PA. Relative importance of surface wettability and charged functional groups on NIH 3T3 fibroblast attachment, spreading, and cytoskeletal organization. J Biomed Mater Res 1998;41:422–30.

[20] Badami AS, Kreke MR, Thompson MS, Riffle JS, Goldstein AS. Effect of fiber diameter on spreading, proliferation, and differentiation of osteoblastic cells on electrospun poly(lactic acid) substrates. Biomaterials 2006;27:596–606.

[21] Patrício T, Domingos M, Gloria A, Bártolo P. Fabrication and characterisation of PCL and PCL/PLA scaffolds for tissue engineering. Rapid Prototyp J 2013;20:145–56.

[22] Ni P, Fu S, Fan M, Guo G, Shi S, Peng J, et al. Preparation of poly(ethylene glycol)/polylactide hybrid fibrous scaffolds for bone tissue engineering. Int J Nanomed 2011;6:3065–75.

[23] Bancroft GN, Sikavitsas VI, van den Dolder J, Sheffield TL, Ambrose CG, Jansen JA, et al. Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proc Natl Acad Sci 2002;99:12600–5.

[24] Liyue L, Zong C, Li B, Shen D, Tang Z, Chen J, et al. The interaction between b1 integrins and ERK1/2 in osteogenic differentiation of human mesenchymal stem cells under fluid shear stress modelled by a perfusion system. J Tissue Eng Regen Med 2014;8:85–96.

[25] Gomes ME, Holtorf HL, Reis RL, Mikos AG. Influence of the porosity of starch-based fiber mesh scaffolds on the proliferation and osteogenic differentiation of bone marrow stromal cells cultured in a flow perfusion bioreactor. Tissue Eng 2006;12:801–9.

[26] Stavenschi E, Labour M-N, Hoey DA. Oscillatory fluid flow induces the osteogenic lineage commitment of mesenchymal stem cells: the effect of shear stress magnitude, frequency, and duration. J Biomech 2017;55:99–106.

[27] Egger D, Krammer M, Hansmann J, Walles H, Kasper C. Dynamic cultivation of human stem cells under physiological conditions. BMC Proc 2015;9(S 9):68.

[28] Su WT, Wang YT, Chou CM. Optimal fluid flow enhanced mineralization of MG-63 cells in porous chitosan scaffold. J Taiwan Inst Chem Eng 2014;45:1111–8.

[29] Bhaskar B, Owen R, Bahmaee H, Rao PS, Reilly GC. Design and assessment of a dynamic perfusion bioreactor for large bone tissue engineering scaffolds. Appl Biochem Biotechnol 2018;185:555–63.

[30] Meinel L, Karageorgiou V, Fajardo R, Snyder B, Shinde-Patil V, Zichner L, et al. Bone tissue engineering using human mesenchymal stem cells: effects of scaffold material and medium flow. Ann Biomed Eng 2004;32:112–22.

[31] Nguyen BNB, Ko H, Fisher JP. Tunable osteogenic differentiation of hMPCs in tubular perfusion system bioreactor. Biotechnol Bioeng 2016;113:1805–13.

[32] Beşkardeş IG, Aydın G, Bektaş Ş, Cengiz A, Gümüşderelioğlu M. A systematic study for optimal cell seeding and culture conditions in a perfusion mode bone-tissue bioreactor. Biochem Eng J 2018;132:100–11.